CHARACTERISATION OF THE MOISTURE CONTENT AND GROWTH OF DONKEY HOOF HORN

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ABSTRACT

The hoof growth, gross moisture content and bound water content of donkeys, mules and ponies kept in a temperate environment in Devon, UK were studied over the course of a calendar year. Samples of donkey hoof from the Donkey Sanctuary were also subject to investigation through immersion in distilled water, and also a series of relative humidity experiments to establish the hydration characteristics of the hoof horn.

The full hoof wall depth and zonal MC% decreased, from the winter months to the summer months for all three species, and then increased once again towards the autumn. The decrease in full hoof wall depth MC% was significant ($p < 0.05$) between the winter and summer months in donkeys and ponies, but not in mules. There were more significant differences between seasonal zonal moisture content for the inner, rather than the outer, hoof wall zones. Contrary to previous theories, it appears that the inner zones of the hoof wall are more sensitive to the effects of season than the outer zones. This may be due to physiological or nutritional factors, rather than being influenced solely by the external environmental humidity or precipitation.

Donkeys had significantly higher ($p < 0.05$) hoof wall depth moisture content than both mules and ponies. Mule hoof wall depth moisture content was on average 2.55% lower than that of the donkey hoof and 5.18% higher than that of pony hoof throughout the seasons. Similar results were obtained for the zonal moisture content analyses, with donkey hoof having the highest, and pony the lowest zonal hoof moisture content. This indicates that the moisture characteristics of mule hoof horn are more like those of donkey, rather than pony hoof, which could have implications for the management of mules in temperate environments.

There were no significant differences ($p > 0.05$) in full hoof wall depth or zonal hoof wall moisture content between locations around the hoof for all three species, although both medial and lateral quarters and heels had higher hoof MC% than the midline dead centre for donkeys, mules and ponies. So although the increased

moisture content may be a major contributory factor in higher elasticity levels in the heels, it is not a significant increase ($p > 0.05$).

When the mean zonal hoof MC% was compared, it significantly increased ($p < 0.001$) in an anterior-posterior direction towards the internal hoof structures, away from the outer hoof wall for donkeys, mules and ponies. The increase in hoof MC% from external zone 1 to internal zone 4 was 12.06% for donkeys, 12.77% for mules and 11.49% for ponies. Interzonal increases were even across the hoof wall depth in the pony hoof, but donkey and mule hoof exhibited large increase in moisture content between the outer zones 1 and 2, and the inner zones 2 and 3.

As with the moisture content results the full hoof wall depth bound water content of donkeys, mules and ponies is at its lowest in summer. The zonal hoof wall bound water content of donkeys, mules and ponies varies minimally between months for the outer two zones, but demonstrates increasing variability between months in zones 3 and 4. The rise in hoof bound water content from external zone 1 to internal zone 4 was on average 0.400 mg/mg for donkeys, 0.357 mg/mg for mules and 0.219 mg/mg for ponies. Pony hoof showed a anterior-posterior hoof bound water content gradient with a gradual increase in bound water content from zone 1 to zone 3 and then a larger increase between zones 3 and 4. The mule hoof bound water content gradient was similar to ponies between zones 1 and 2. It then showed distinct divisions with larger increases between zones 2 and 3, and zones 3 and 4, which was more characteristic of the donkey hoof bound water content gradient. This may indicate that the water binding characteristics of the mule hoof are some kind of hybrid between those of the pony and the donkey.

In pony and mule hoof there was no significant difference ($p > 0.05$) between hoof growth rates (mm/day) for between months. Donkey hoof experienced a significant drop in hoof growth rate between September and November ($p < 0.05$). The mules and ponies displayed cyclical spurts in hoof growth rates, with increased growth rates in February, May and September. There was no significant difference ($p > 0.05$) in hoof growth rates around the circumference of the hoof for any of the species. Mule hoof had the highest, and donkey the lowest, hoof growth rate.

The mule hoof wear rate was higher than for both donkey and pony at all locations. In all three species the midline dead centre hoof wear rate was significantly higher ($p < 0.05$ to $p < 0.001$) than the quarters and heels

There was a significant effect ($p < 0.05$) of hoof wall location on net hoof growth/wear for the midline dead centre sampling site for all species. The months of February, April and November have the lowest net hoof growth/wear rate for all three species. All species appear to display alternating periods of elevated growth rate, followed by elevated wear rate, producing cyclical fluctuations in the net hoof growth/wear rates.

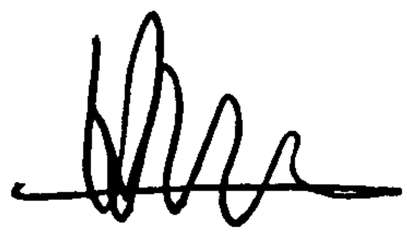
General linear model analysis showed no significant interactions ($p > 0.05$) between hoof replacement time (days) and species, farms, gender, hoof pigment, or hoof wall locations. Donkey hoof replacement time was greater than that of both mules and ponies due to the slow hoof growth rate of donkey hoof horn.

Donkey hoof wall zones 1 and 2 displayed similar desorption isotherms and similar sorption isotherms, as did donkey hoof wall zones 3 and 4. This would indicate an 'inner' and 'outer' hoof wall depth split with regard to hydration properties of the hoof horn, as there were noticeable differences between the desorption and sorption isotherms for the outer hoof wall zones (1 and 2), and the inner hoof wall zones (3 and 4).

Donkey hoof horn samples can be immersed in distilled water and reach equilibrium mass, gaining between 3-6 mg/100mg mass, within 3hrs of first being immersed. Donkey hoof wall zones 1 & 2 consistently displayed a 5mm/100mm increase in sample width, associated with the outer full hoof wall circumference. There was no significant change in sample height or length dimensions, which would indicate no significant change in hoof wall height or hoof wall depth of an entire donkey hoof *in vivo*.

DECLARATION

I hereby declare that the work presented in this thesis was carried out by myself and has not been presented to any other University, or published elsewhere, with the exception of the papers published in peer reviewed journals which have been included in the Appendices.



Heidi Wealleans 23 July 2008

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ABBREVIATIONS, SYMBOLS AND GLOSSARY OF TERMS USED IN THE THESIS

Abbreviation	Term
°C	Degrees Centigrade
CE	Cell Envelope
cm	centimetre(s)
D	Donkey
DSC	Differential Scanning Calorimetry
EPOM	Evening Primrose Oil Mixture
FHMC	Fully Hydrated Moisture Content
FMC	Fresh Moisture Content
g	Gram (s)
H	Horse
HGR	Hoof Growth Rate
HH	Heel Height
HL	Hoof Length
HPA	Hoof Pastern Axis
HRT	Hoof Replacement Time
HW	Hoof Width
HWA	Hoof Wall Angle
HWD	Hoof Wall Depth
HWH	Hoof Wall Height
HWR	Hoof Wear Rate
IF	Intermediate Filaments
IFAP	Intermediate Filament Associated Proteins
IT	Intertubular Horn
°K	Degrees Kelvin
kg	Kilogram(s)
LH	Lateral Heel
LQ	Lateral Quarter
M	Mule
MC	Moisture Content
MDC	Midline Dead Centre
mg	Milligram(s)
MH	Medial Heel
mm	Millimetre(s)
MQ	Medial Quarter
μm	Micrometre(s)
n	Sample Number
P	Pony
P_2O_5	Phosphorous Pentoxide
RH	Relative Humidity
SEM	Standard Error of Mean
StDev	Standard Deviation
T	Tubular Horn

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CHAPTER 1 – INTRODUCTION AND THE ANATOMY OF HORSE AND DONKEY HOOVES

1.1 Introduction

There are approximately 41 million donkeys and 55 million horses worldwide (FAOSTAT data, 2006). In the United Kingdom, there are approximately 10,000 donkeys (Starkey & Starkey, 1997) and 1.2 million horses (BETA, 2006). In contrast to the developing world, where the donkey is mainly used for work and is vital to many families, the majority of UK donkeys are kept for pleasure as pets, although there are about 850 working donkeys (Anon, 2002), that are used for riding and driving.

Although they are often treated by vets as small horses (Svendsen, 1997), the donkey (*Equus asinus*) is in fact, a distinctly different species to the horse (*Equus caballus*), and evolved as such 1.5million years ago (Groves, 1974). *Equus caballus* adapted to survive on the open grassland plains of Eurasia, whereas *Equus asinus* evolved to survive in arid conditions with sparse vegetation, often travelling miles over difficult terrain, including rocky outcrops, to obtain food.

This parallel evolution of the horse and the donkey has produced many anatomical differences, as well as some similarities, between the species. Divergent evolutionary development has led to differences in limb conformation, particularly in the hoof.

Mules, the hybrid offspring of a donkey jack and a horse mare (Burnham, 2002) , are of economic importance in 3rd world countries, and have a wide variety of uses as draught or pack animals. There are approximately 12.5 million mules in the world (FAOSTAT data, 2006). Limited studies have been carried out on the mule, which is surprising considering their widespread use and importance in Africa, Asia and South America, where millions of people depend on mules for everyday life. Matthews *et al.*, (1997) reported differences in the physiology and behaviour of mules and donkeys, when compared to horses, which included drug distribution and pharmacology. These

differences would indicate that the physiological processes which occur in mules and donkeys probably extend to the complex structure and biochemistry of the hoof. No study describing detailed hoof parameters of the mule was found in the literature review, although Jackson (1993) reported subjective observations of high quality hooves in feral mules in the Australian outback. Burnham (2002) reported various anatomical differences between the donkey and the mule, as well as the presence of more prominent ergots in the donkey, when compared to those of the horse, although no in depth assessment of hoof structure was made.

Pollitt (1995) described the equid hoof as a "miracle of bioengineering". Its primary functions are to provide a protective capsule of horn surrounding the sensitive tissues of the foot and to dissipate the forces of locomotion (Dyhre-Poulsen *et al.*, 1994). The anatomical differences between horse and donkey hoof structure signify possible variation in locomotion and function, as it is thought that the shape and mechanical properties of the hoof affect the natural functioning of the hoof wall (Leach, 1980).

For this reason it is important to study the structure/function relationship of the hoof horn material. Isolated studies of donkey foot anatomy have been made by Dogeur (1943), Hifny & Misk (1983) and Alexander & Dimery (1985), who also made structural observations. However, more recent investigations have furthered the understanding of the relationship between the structural and functional properties of the hoof capsule (Hopegood, 2002). This was achieved by characterisation of the structure of the donkey hoof wall, subjecting hoof samples to mechanical tests to provide information about material properties and then producing a finite element analysis model of the donkey hoof capsule (Collins *et al.*, 1998) (Newlyn *et al.*, 1998).

A model of hoof function is only as good at predicting performance as the data that is supplied to inform it. Since moisture content of horn directly affects the mechanical properties of the tissue, it is essential to have accurate information about this parameter in order to acknowledge normal states and abnormal states, and to predict the practical consequences for hoof function. There are queries as to whether the hoof moisture content differs between species, between anatomical locations around the circumference

of the hoof wall, and whether it varies at different times of year. Once this information is known for healthy donkey, mule and pony hoof horn, it could be used as a bench mark to assess the effect that disease may have on the moisture content, and therefore hoof function of diseased donkeys, mules and ponies. Kempson & Cambell (1998) used horseradish peroxidase to trace water permeability within hoof horn, and found a definite barrier to the permeability of the hoof wall. They also established that poor quality diseased horn was more susceptible to penetration by the tracer than healthy good quality hoof horn.

Any differences between the hoof growth and wear rates of the two species have also yet to be investigated. It is still unknown whether there is variability in the hoof growth or wear rate between anatomical locations around the circumference of the hoof wall, and whether growth and wear vary at different times of year. Hoof growth and wear rates have huge implications for the functionality of the hoof, either for maintaining adequate hoof wall to support the animal during locomotion, or for growing out a crack or a lesion. Fowler (1995) reported that the hoof horn growth rate in unshod donkeys is sufficient to cope with the work that they undertake in the UK, even on metalled roads. If the growth and wear rates for healthy donkey, mule and pony hoof horn could be ascertained, this would provide scientific evidence to either support or refute the deduction of Fowler (1995). They could also then be used to predict the time taken for hoof lesions to grow out from the coronary band to the bearing border.

The terms "bound" and "free" water may be used to describe the state of water in many biological substances which retain moisture. Bound water is that which remains unfrozen once a material is cooled below 0^{o}c, whereas free water will freeze at temperatures of 0^{o}c or below. For example, in the keratin of human fingernails “free” water is incorporated between the keratin filaments and there is also water more tightly “bound” in keratin complexes (Schulz *et al.*, 2002). In the analogous case for the hoof material, the hydration and dehydration dynamics can be investigated by dimensional changes in water soaked samples, by quantifying bound and free forms in samples and by sorption isotherms when hoof horn is equilibrated at a particular relative humidity.

In order to address parameters mentioned above, it is important to understand the basic structural features of the hoof. Section 1.2 outlines the important issues in describing the hoof anatomy and where appropriate, highlights the differences between horses and donkeys. Unfortunately no post mortem mule hoof samples were available for anatomical comparison with donkey and horse hooves, as there were no mule deaths at the Donkey Sanctuary during the experimental time period, and Burnham (2002) made no assessment of mule hoof anatomy.

1.2 Differences between donkey and horse hoof capsules

Gross hoof structures in donkeys and horses share many anatomical features, although there are also significant differences that are apparent visually. The basic hoof and foot structures are present in both donkeys and horses, and are shown in Figure 1.2.1.

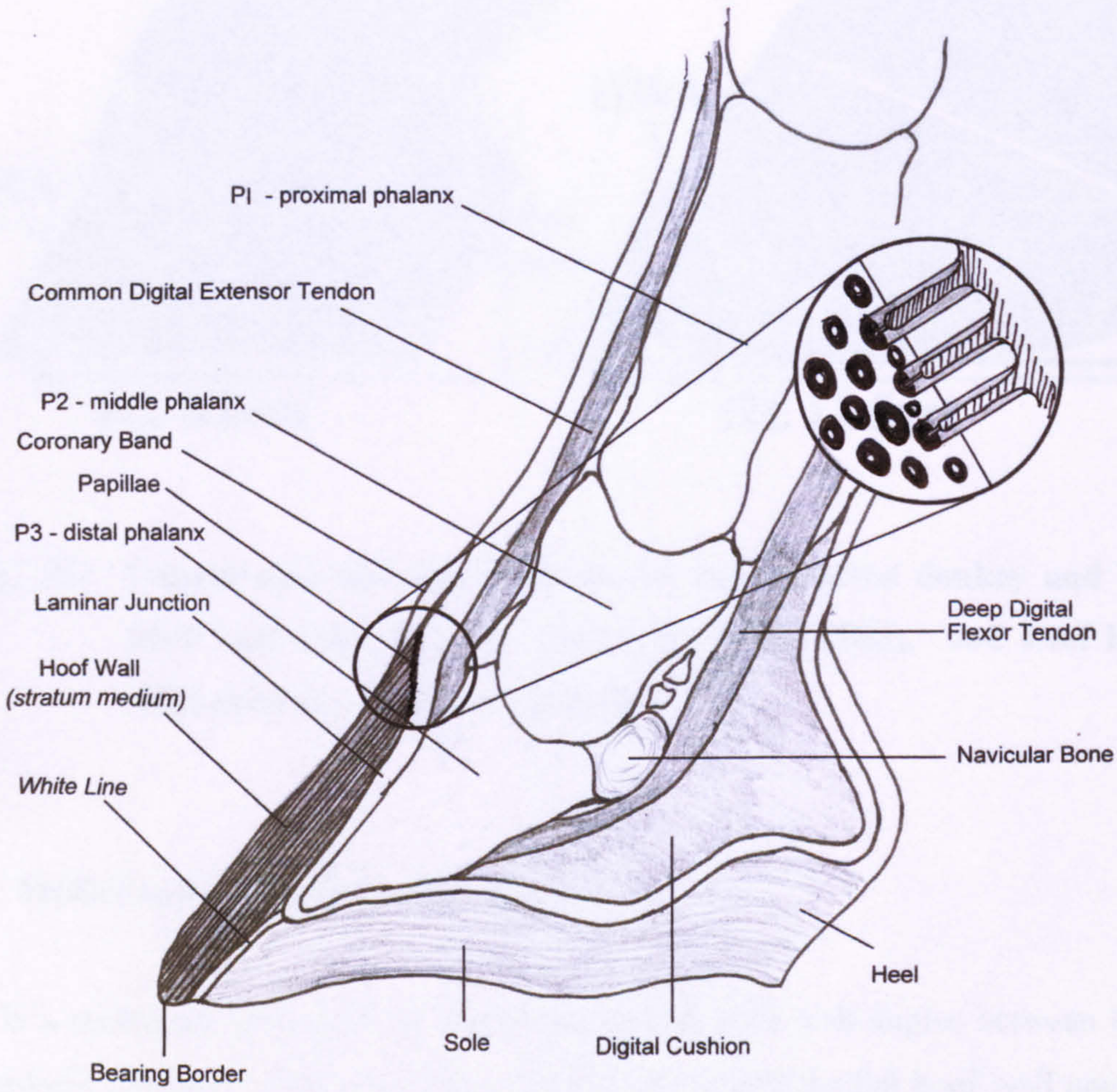


Figure 1.2.1 Structures of the equid hoof and foot (author's own illustration)

1.2.1 Hoof wall length and angles

There are noticeable differences in hoof wall length (HWL) and dorsal hoof wall angle (HWA) between horses and donkeys (Figure 1.2.2). Horses have hoof wall angles of approximately 45° in the forefeet and approximately 50° in the hind feet (Stump, 1967), whereas the donkey hoof is slightly more upright with angles of approximately 55° and approximately 62° respectively (Hifny & Misk, 1983). It may be that these differences can be attributed to the hardness of the ground surface in drier countries, which could have a bearing on the hoof function in terms of the dynamics of locomotion and also the hoof wear rate.

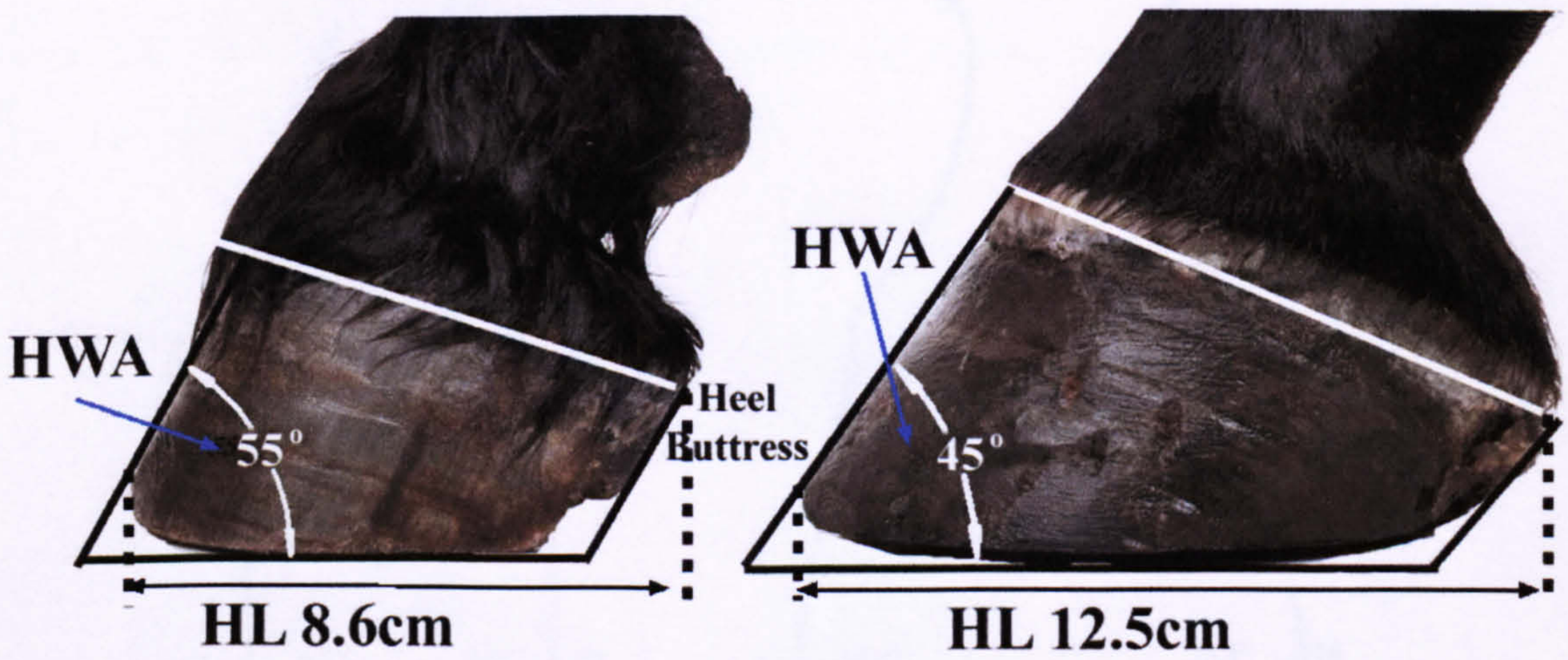


Figure 1.2.2 Lateral view left fore hoof illustrating reported donkey and horse hoof wall angle (HWA) (Hifny & Misk, 1983), and hoof length (HL) (author's own photograph)

1.2.2 Medial and lateral hoof wall angles

There is a noticeable difference in lateral and medial hoof wall angles between horses and donkeys (Figure 1.2.3), with horses having lateral and medial hoof wall angles of approximately 78°, whereas the donkey hoof is more upright with an approximate

lateral angle of 91.5° and a medial angle of 89° respectively (figures calculated from external angle measurements by Hifny & Misk, 1983).

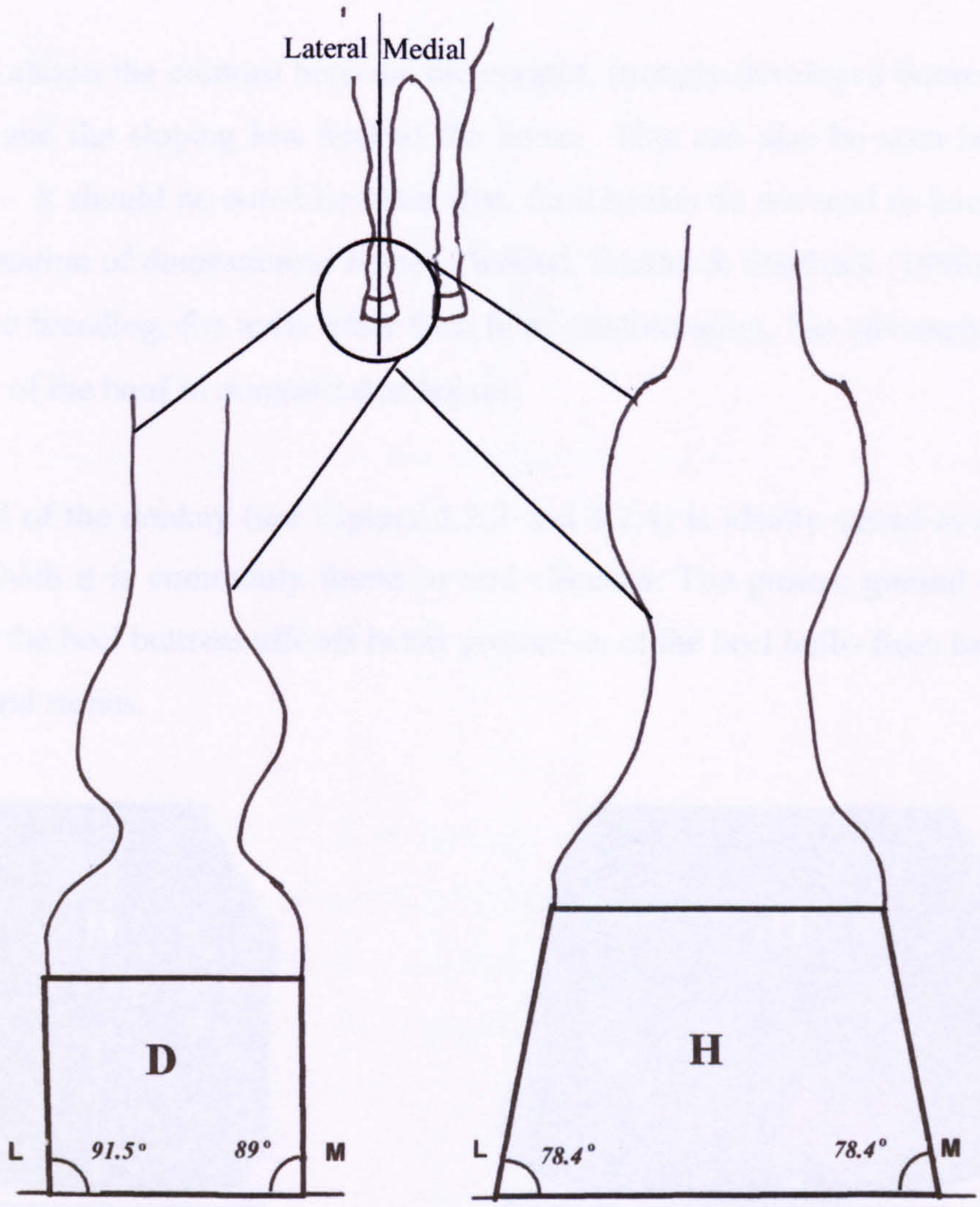


Figure 1.2.3 Dorsal schematic view of lateral (L) and medial (M) hoof angles for donkey (D) and horse (H) right fore hooves as reported by Hifny & Misk (1983) (author's own illustration)

The precise effects of these differences in hoof wall angles between horse and donkey capsules on the mechanics of the hoof are unknown. However, they are thought to lead to varying degrees of compressive and tensile forces within the hoof capsule during loading. Newlyn *et al.* (1998) modelled deformation under load in the donkey, and

found that there is likely to be increased axial resistance leading to decreased heel expansion compared with horses.

Figure 1.2.4 shows the contrast between the upright, strongly developed buttress heel of the donkey and the sloping low heel of the horse. This can also be seen laterally in Figure 1.2.2. It should be noted however that, feral horses do not tend to have the low heel conformation of domesticated horses. Indeed, Caddel & Stanback (1998) believed that selective breeding, for traits other than hoof conformation, has adversely affected the integrity of the hoof in domesticated horses.

The tall heel of the donkey (see Figures 1.2.2 and 1.2.4) is ideally suited to the rough terrain in which it is commonly found in arid climates. The greater ground clearance provided by the heel buttress affords better protection of the heel bulbs from bruising by hard rocks and stones.

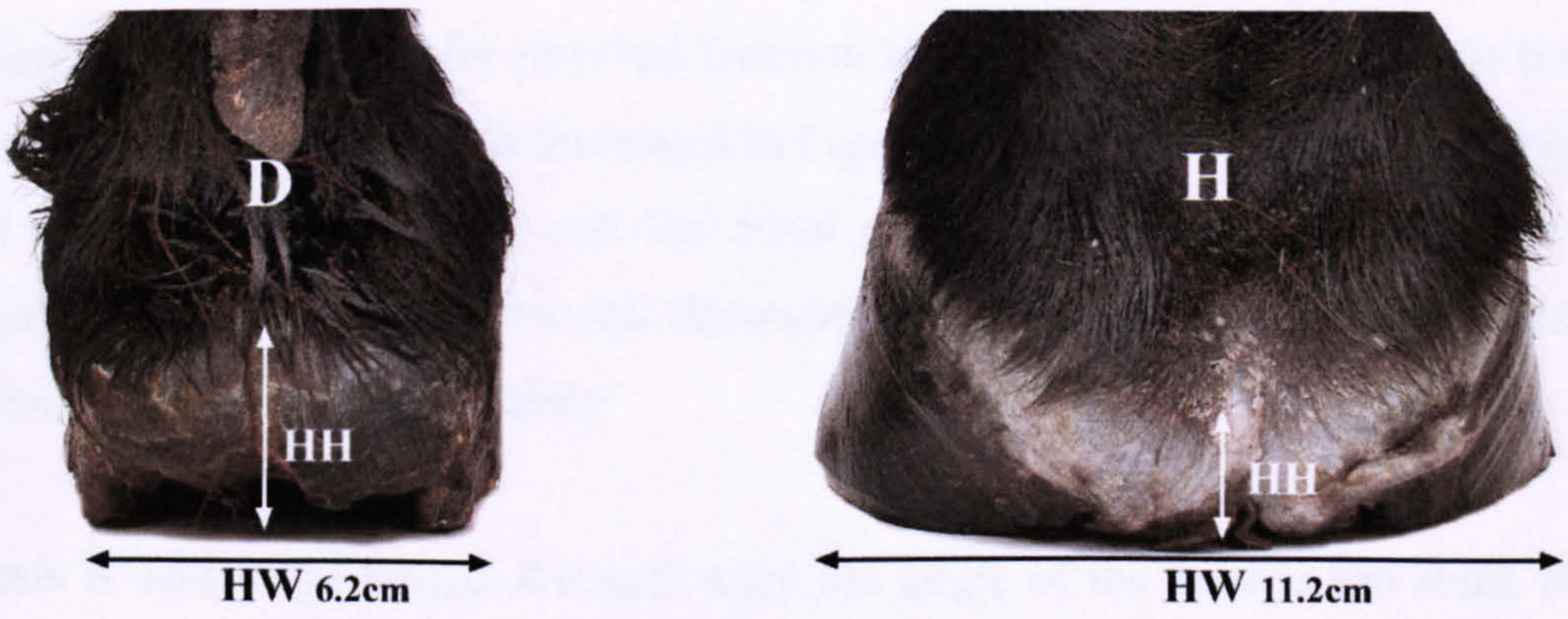


Figure 1.2.4 Donkey (D) and horse (H) front feet - palmar view illustrating hoof width (HW) and heel height (HH) (example measurements from Dogeur [1943]) (author's own photograph)

The differences in the lateral capsule appearance, such as those seen in Figure 1.2.1, can be expressed by the ratio of the heights of the wall at the toe, quarters and heels (Table 1.2.1).

Table 1.2.1 Wall height ratios for donkeys and horses

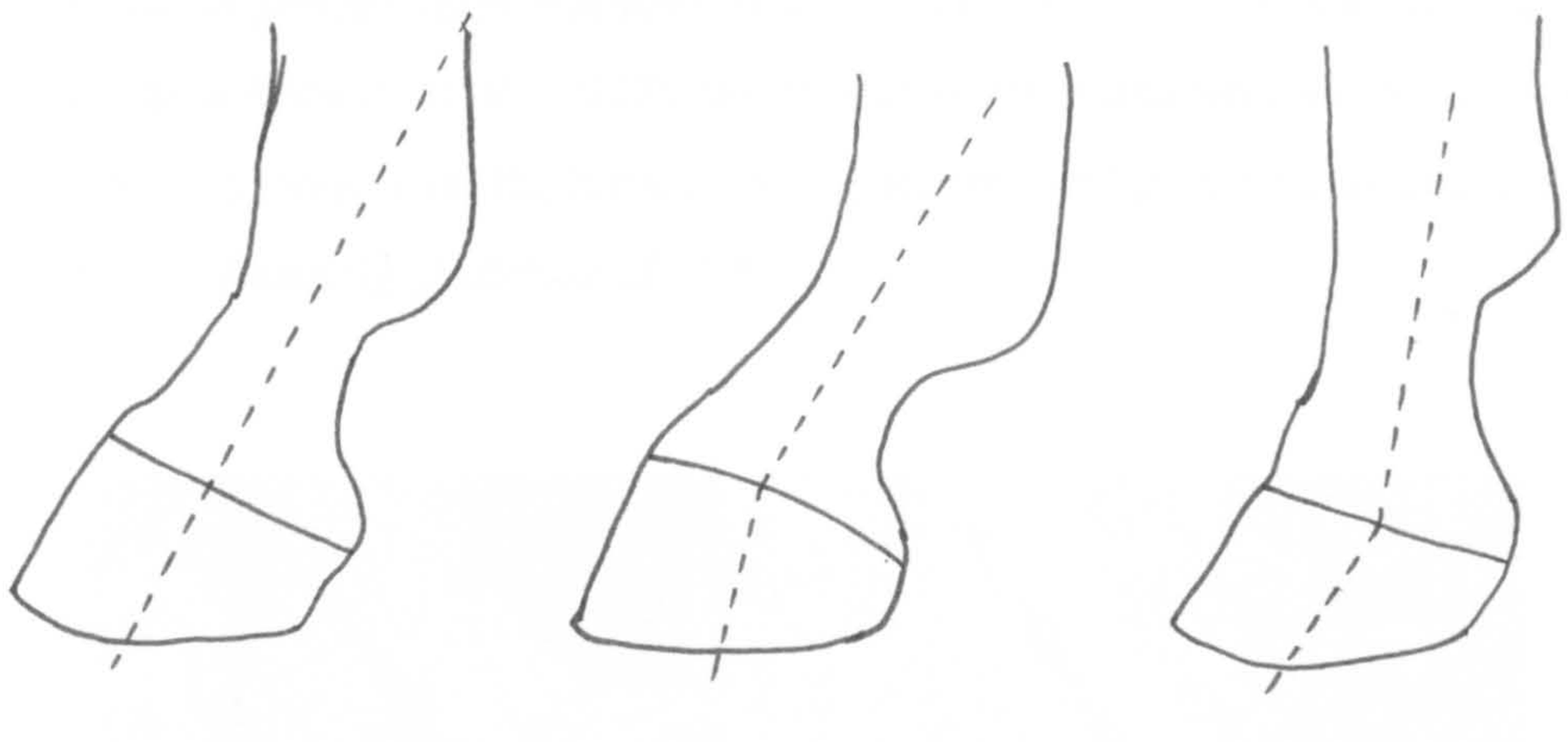
Species	Forefoot	Hind foot
Donkey (Hifny & Misk, 1983)	3 : 3 : 1.5	2 : 1.5 : 1
Horse (Getty, 1975)	3 : 2 : 1	2 : 1.5 : 1

1.2.3 Hoof pastern axis

The hoof pastern axis (HPA) is the line through the centre of the distal, middle and proximal phalanxes (Figure 1.2.5). Alignment of the dorsal hoof wall angle and pastern angle (Figure 1.2.5a) has been an objective of farriers and veterinarians for centuries (Gibson & Stashak, 1990) in order to minimise the internal stresses being placed on the foot.

The optimal conformation for physical function in the horse would result in a straight HPA (Page & Hagen, 2002), as illustrated in Figure 1.2.5a, parallel with the angle of the dorsal aspect of the hoof wall and the distal phalanx. This would place the least amount of stress on the tendons and ligaments of the lower leg and would therefore reduce the chances of tendon injury.

The axis is said to be broken forward when the angle of the hoof is too steep for its associated pastern (Balch *et al.*, 1997) (Figure 1.2.5b). In the horse this is usually when the heels are too high and the toe is too short. Conversely, hooves are described as broken backward when the angle is too sloping for its associated pastern (Figure 1.2.5c), which occurs when the heels are too low and/or the toe has been allowed to grow excessively long in the horse.



a **b** **c**

Figure 1.2.5 Ideal hoof pastern axis (a) (HPA), and broken forwards (b) and broken backwards (c) HPA (author's own illustration)

It is known that the growth and wear of the hoof between regular trimmings affects the moment about the distal interphalangeal joint, between the middle and distal phalanges (Moleman *et al.,* 2006). This indicates that there is a compensatory mechanism in the distal interphalangeal joint for the change in hoof conformation. This leads to increased loading of the deep digital flexor tendon (Figure 1.2.1) via an increase in the dorsal angle of the fetlock joint, and an associated decrease of the dorsal angle of the hoof wall (Moleman *et al.,* 2006). Thus, growth of horn between trimmings is thought to have a significant effect on the loading of the hoof and the movement of the horse.

Eliashar *et al.,* (2004) correlated the forces applied to the hoof wall with the changes in the ratio of heel to toe heights, and the angles of the distal phalanx. This thereby infers that the forces applied to the hoof would be affected by the growth and wear rates of the hoof horn as these could affect the ratio of heel to toe height.

There is considerable debate as to the optimal hoof pastern axis in the donkey. Donkeys have a prominent band of soft tissue and hair that bulges above the coronary band (Figure 1.2.6a) which can make estimating the hoof pastern axis difficult, and give the

appearance of a broken forward HPA. Some equine researchers prefer to use the angle of the scapula to predict HPA because of the variability of soft tissue thickness at the pastern in horses (Emery *et al.,* 1977), but this in itself is inconsistent, as the scapula is often obscured by muscle on the horse, and the scapula and pastern can sometimes form different angles naturally (Balch *et al.,* 1991).

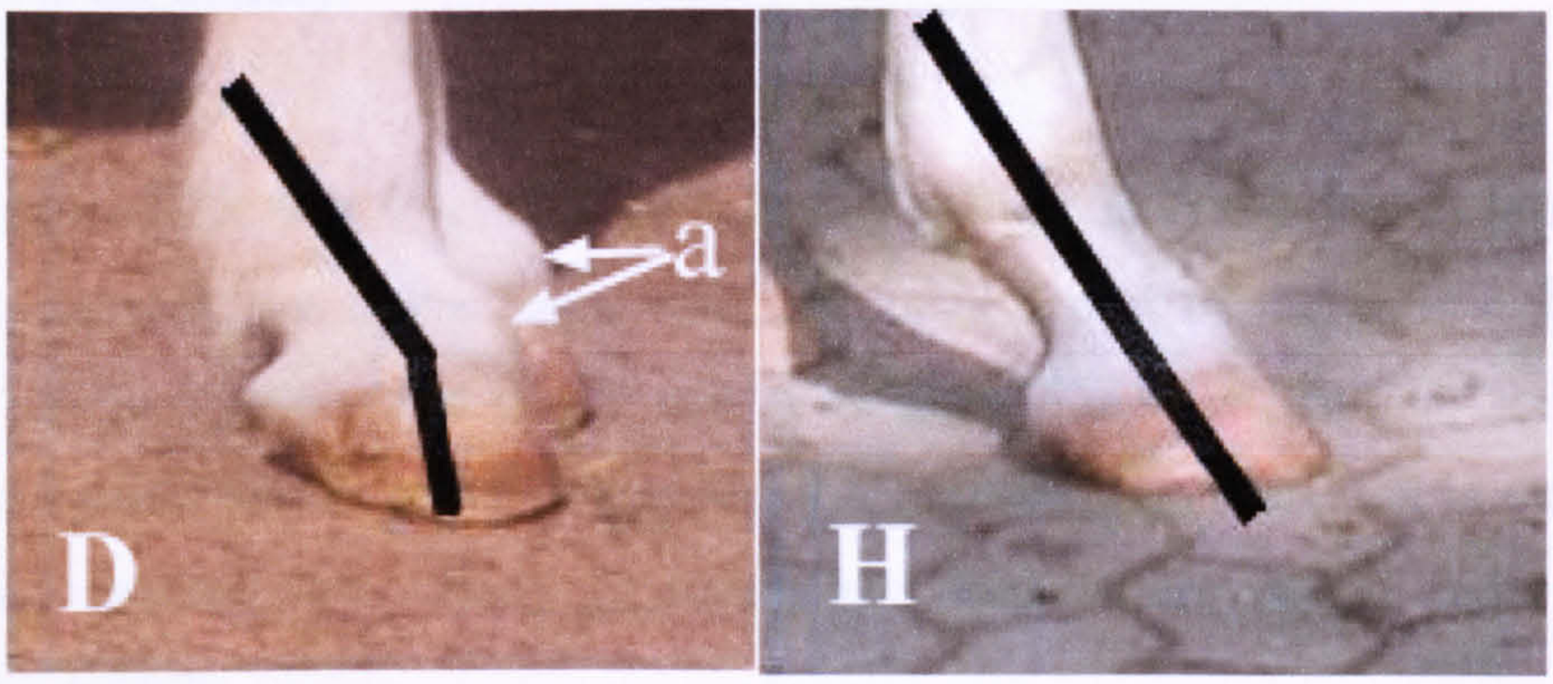


Figure 1.2.6 Weight bearing donkey and horse front hoof - lateral view HPA (author's own photographs)

It is hardly surprising that there is disagreement about the true hoof pastern axis in the donkey. Even with horses, individual variations and breed conformation can cause confusion when assessing the hoof pastern axis (Balch *et al.,* 1991). Donkey hoof has appreciably different conformation to horse hoof, so the situation is confused further.

It is often postulated that donkeys should have the ideal (straight) HPA, but this theory was developed for the horse to create optimum stress and strain situations with regard to the hoof capsule, joints, ligaments and tendons of the lower leg (Reilly, 1997).

The legs of the donkey and horse have evolved to maintain each species in its natural environment. Both species have 'fight or flight' instincts, although the horse tends to rely primarily on the flight instinct, and is capable of travelling large distances at high speed to avoid predators (Jackson, 1997a). Whereas the donkey tends to stand and fight, preferring to kick to defend itself if necessary, and rarely breaking out of a walk or trot voluntarily (French, 1997).

A broken forward axis is seen in the horse when the heels are too high, but the donkey naturally has a high heel (as has been shown in Figures 1.2.1 and 1.2.3). This could add weight to the argument that the donkey naturally has a broken forward HPA. In the horse a broken forward HPA would result in excess stress on the lower limb joints, and concussion to the joints would probably be increased. Such concussion problems are rarely seen in the donkey.

The only dependable way to establish the hoof pastern axis of the donkey would be to take radiographs of the lower limb, with the dorsal aspect of the hoof wall marked vertically on the midline dead centre with a metal wire marker (Collins, 2004). This would then further the understanding of lower leg anatomy and function in the donkey, and indicate that the lower limbs of horses and donkeys are indeed under different mechanical stresses.

1.2.4 The sole

Donkey hooves have a distinct oval solear shape (Hifny & Misk, 1983) compared to the rounder appearance of the horse sole. Dogeur (1943) measured the length (referred to as hoof length, Figure 1.2.1) and width (referred to as hoof width, Figure 1.2.3) of hooves from both species and found the donkey hooves to be relatively elongated, whereas horse hooves were rounder in appearance. There is a distinct heel buttress (Figure 1.2.1), and there is often a flare at the heels (Fowler, 1995).

The surface of the sole is concave with the deepest part of the concavity near the apex of the frog (Schummer *et al.*, 1981). It has been reported that the donkey has a greater thickness of sole than the horse (Hifny & Misk 1983, Dogeur 1943).

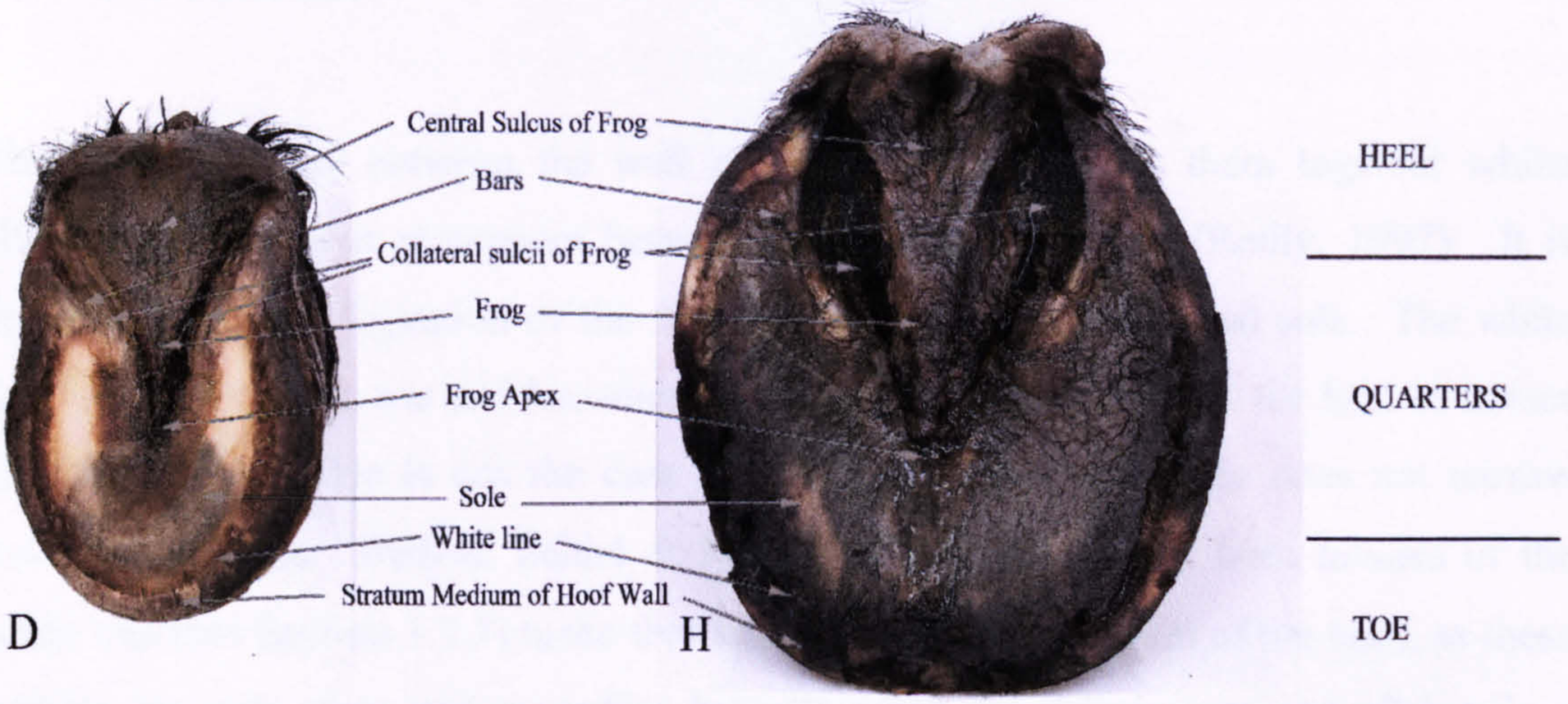


Figure 1.2.7 Donkey (D) and horse (H) front feet - ventral view illustrating difference in solear shape (author's own photograph)

According to Fowler (1995), in the UK, because of ambient climatic conditions, donkeys have quite a large area of weight bearing sole, especially near the toe (Fowler, 1995). Donkeys working abroad in arid conditions do not display the large area of weight bearing sole (Trawford, 2002, personal communication). This is similar to the finding of Hayes (1969) where horses had more concave, and therefore less weight bearing, soles when living in arid areas. The flat soles seen in the UK are more prone to bruising and lameness. Conversely, desert region horses which have lived in the wild for several generations have developed a hoof smaller in width and more concave in sole (Emery *et al.*, 1977). This greater ground clearance affords better protection of the sole and frog from bruising by hard rocks and stones.

1.2.5 The frog

In both donkeys and horses, the frog is easily discernable as the 'V' shaped structure on the ventral surface of the hoof occupying the angle bounded by the bars and sole, its apex pointing towards the toe of the hoof (Figure 1.2.7).

1.2.6 The white line

The white line lies between the wall and the sole, and binds them together whilst allowing independent movement between the sole and the wall (Reilly, 1997). It is formed by the interdigitation of the sensitive laminae of the wall and sole. The white line is thickest at the toe and becomes progressively thinner towards the heel in horses (Kainer, 1989). This is not the case in donkeys, as the white line does not narrow towards the heels. Budras, Schiel & Mulling (1998) found that horn tubules of the white line (see Section 1.2.7) make that structure the weakest point of the hoof, as these tubules are very short and ascending bacteria reach the dermis (corium) after only a short passage. Potentially, the white line could serve as a mechanical buffer zone between the rigid hoof wall and bone and laminar tissues, and could assist the feet of horses in transmitting a load through the tissues and prevents the most susceptible tissues from becoming damaged (Wagner *et al.*, 2001).

1.2.7 The wall

The hoof wall is usually divided up into the toe, quarter, and heel regions (Figure 1.2.7). The main function of the wall is to bear the weight of the equid. The wall reflects in at the heels to form the bars (Figure 1.2.7). The equid hoof, along with the skin and other appendages, consists mainly of keratinocytes. The proliferating cells are chiefly located in the basal cell layer and the differentiating cells are arranged in several suprabasal cell layers where they undergo an orderly sequence of maturation described by the term keratinisation (Wattle, 1998). For a more detailed description of keratinisation, see Section 1.2.9. The wall grows distally from the coronary band, with the keratinocytes arranged into a tubular and intertubular horn, which can be viewed in cross section. The hoof wall is constructed of three layers of horn (Figure 1.2.8) with a thin external layer (*stratum externum*), a middle layer (*stratum medium*) and an internal laminar layer (*stratum internum*).

The *stratum internum* (Figure 1.2.8) is composed of primary and secondary epidermal lamellae that interdigitate with complementary primary and secondary dermal lamellae (Leach & Oliphant, 1983). These connect the heavily keratinised hoof wall, via a basement membrane, to the dense connective tissue of the third phalanx (Pollitt, 1994). There are approximately 600 lamellae in the horse, and 350 lamellae in the donkey (Hifny & Misk, 1983). The lamellae provide a large surface area and allow the wall to effectively deal with the multidirectional forces that occur during weight bearing and locomotion (Reilly, 1997).

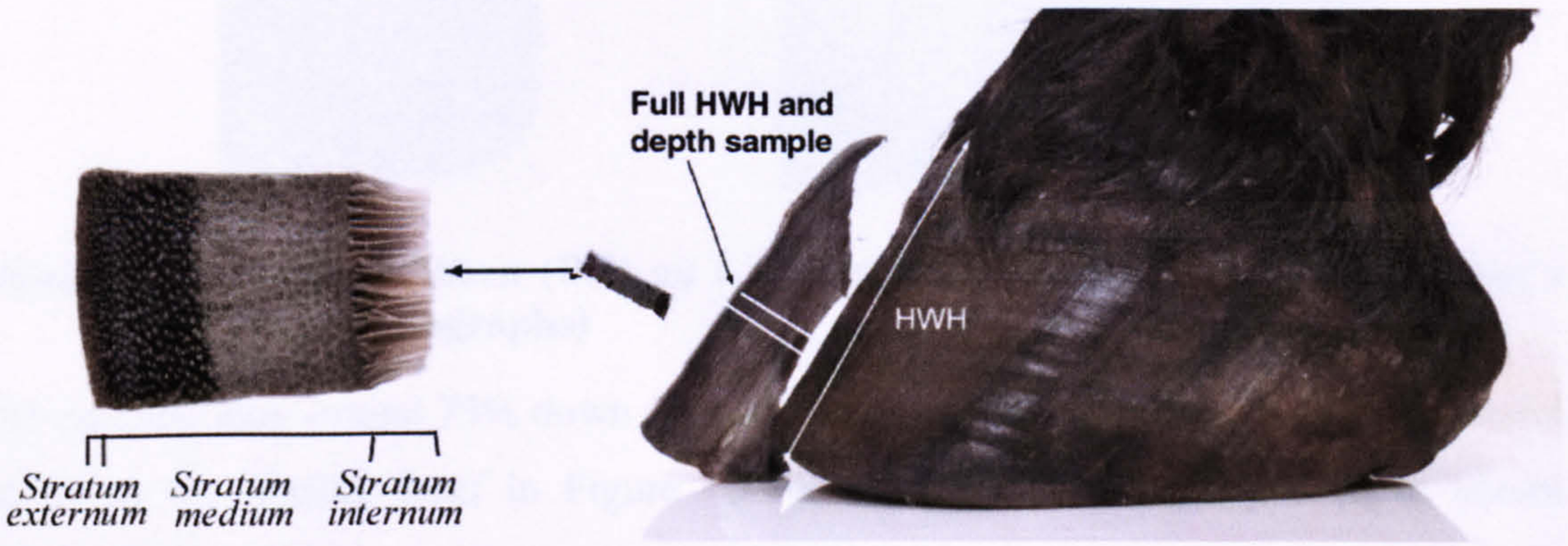


Figure 1.2.8 Donkey hoof wall depth (HWD) segment showing the three layers of hoof horn (author's own photograph)

The *stratum medium* is the largest portion of the hoof wall (Turner, 1997) (Figure 1.2.8), and is derived from mitotically active epidermal cells in the proximal region of the wall, termed the coronary border, interior and subjacent to the periople (Leach, 1980; Marshall, Orwin & Gillespie, 1991) (Figure 1.2.1).

The *stratum externum* includes the periople, which is a band of soft, tubular horn that passes through a keratohyaline stage in its cornification, with the end result being a 'soft' keratin. It function is to 'cement the skin to the hoof' (Smith, 1921) and is thought to prevent undue evaporation from the horn beneath (Reilly *et al.*, 1998a). In donkeys the perioplic groove broadens at the heels and fuses with the frog (Hifny & Misk, 1983), but in horses the perioplic groove merges with the coronary groove (Getty, 1975).

The amount of hoof wall covered by the periople depends on the abrasiveness of the equid's environment. In stabled horses it may extend halfway down the hoof wall (Pollitt, 1995), but Figure 1.2.9 shows a horse with only the top 25% of the hoof wall covered with perioplic horn.

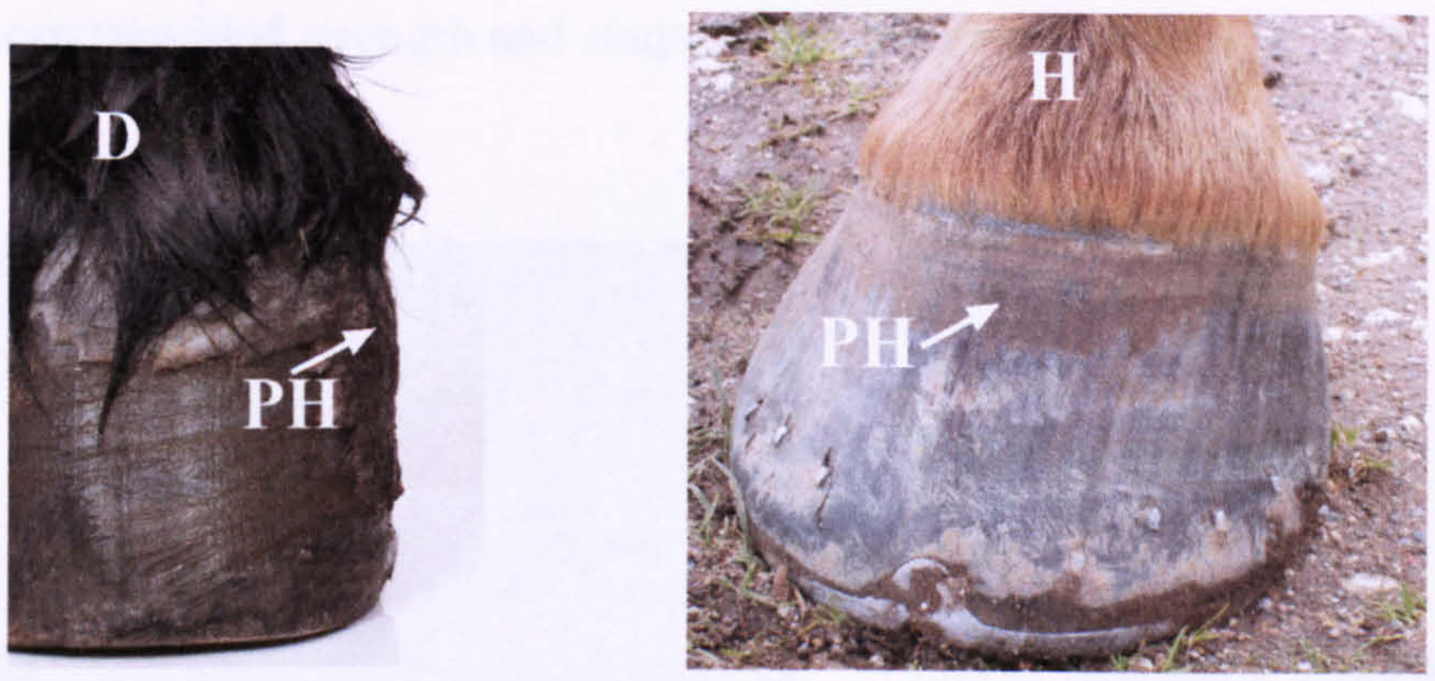


Figure 1.2.9 Perioplic horn (PH) on a donkey (D) and horse (H) hoof (author's own photographs)

The periople may extend 75% down the hoof wall in donkeys in the UK (see the lateral aspect of the donkey hoof in Figure 1.2.9), although this is rarely seen in horses (personal observation).

1.2.8 Tubular and intertubular horn

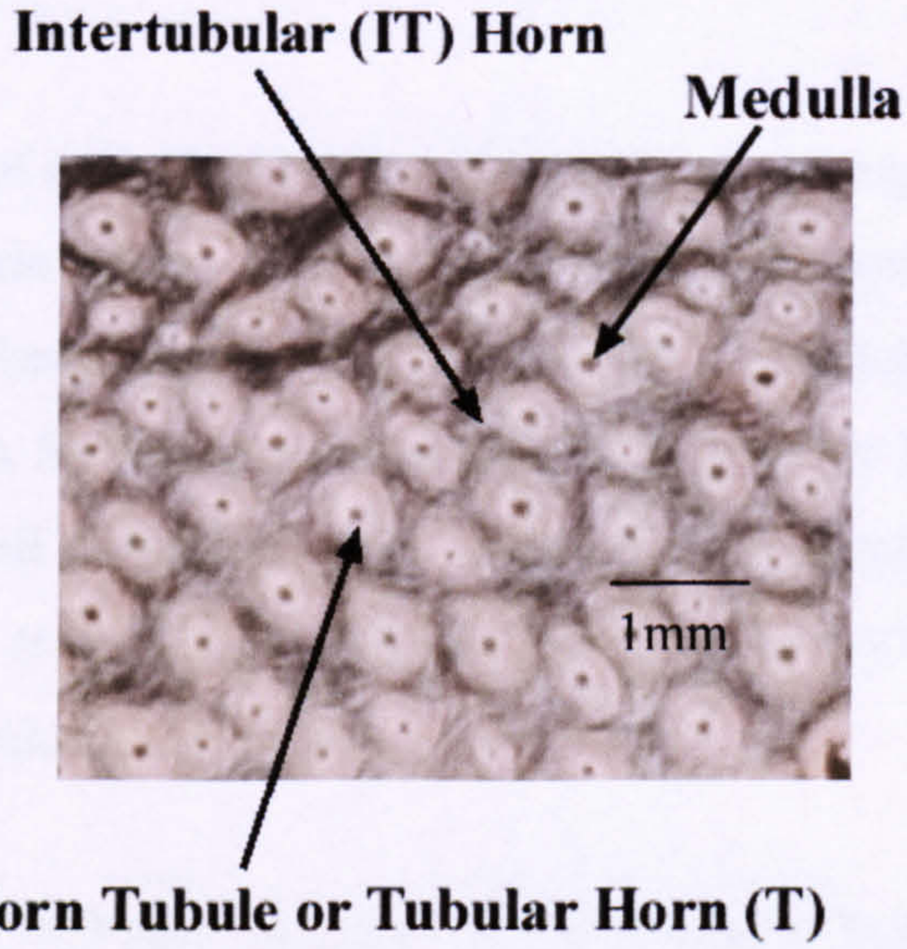


Figure 1.2.10 Tubular and intertubular horn (donkey hoof) (author's own photograph)

The keratinocytes of the *stratum medium* are arranged into tubular and intertubular horn. One of the earliest known records of the presence of tubular and intertubular horn in the *stratum medium* of the hoof wall is that of Lungwitz & Adams (1897). Balch *et al.*, (1997) believed that the orderly proximodistal arrangement of tubular and intertubular horn provided strength and shape to the hoof wall.

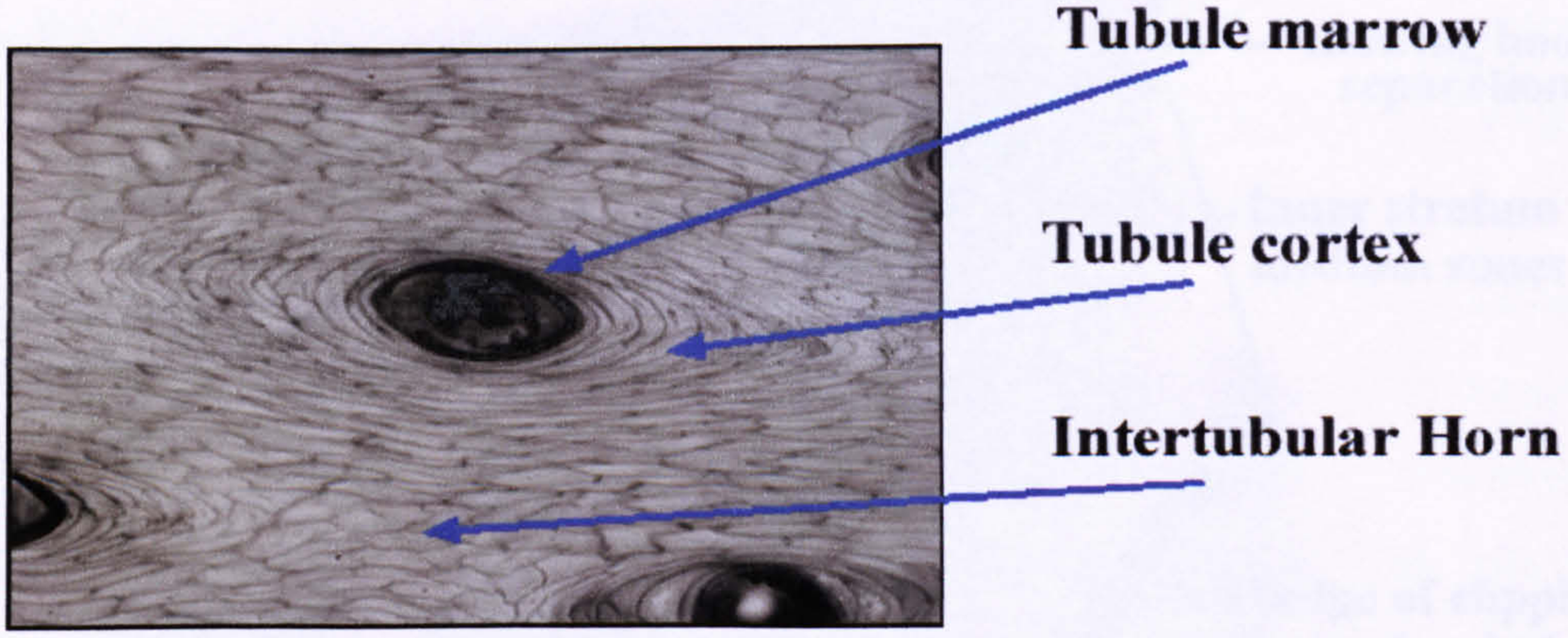


Figure 1.2.11 Hoof wall tubule (x 200) (author's own photograph)

Bertram and Gosline (1987) believed that the tubules had a significant effect on the manner in which forces are distributed in the hoof wall. Nickel (1939) also suggested that the intertubular horn distributed the stresses to the tubules, which acted as struts in the hoof wall.

The keratinocytes of individual hoof wall tubules are arranged around a central hollow medulla in concentric layers. Keratinocytes generated between the tubules mature into intertubular horn – keratinised extracellular matrix - in which the tubules are embedded. Intertubular horn is formed at right angles to the tubular horn (Figure 1.2.11) – this makes the hoof wall stronger at right angles to the direction of the tubules (Pollitt, 1998). Thomason *et al.,* (1992) reasoned that the microarchitecture of the hoof wall is an adaptation to mechanical function.

Thomason *et al.,* (1992) also suggested that intertubular horn contributed more to hoof strength, stiffness and fracture toughness than the tubules, with the tubules three times more likely to fracture. Leach (1980) conducted compressive testing and also found

that intertubular horn accounted for a major part of the mechanical behaviour of the hoof wall, with the tubules providing reinforcement to the structure of the horn (Kasapi & Gosline, 1997).

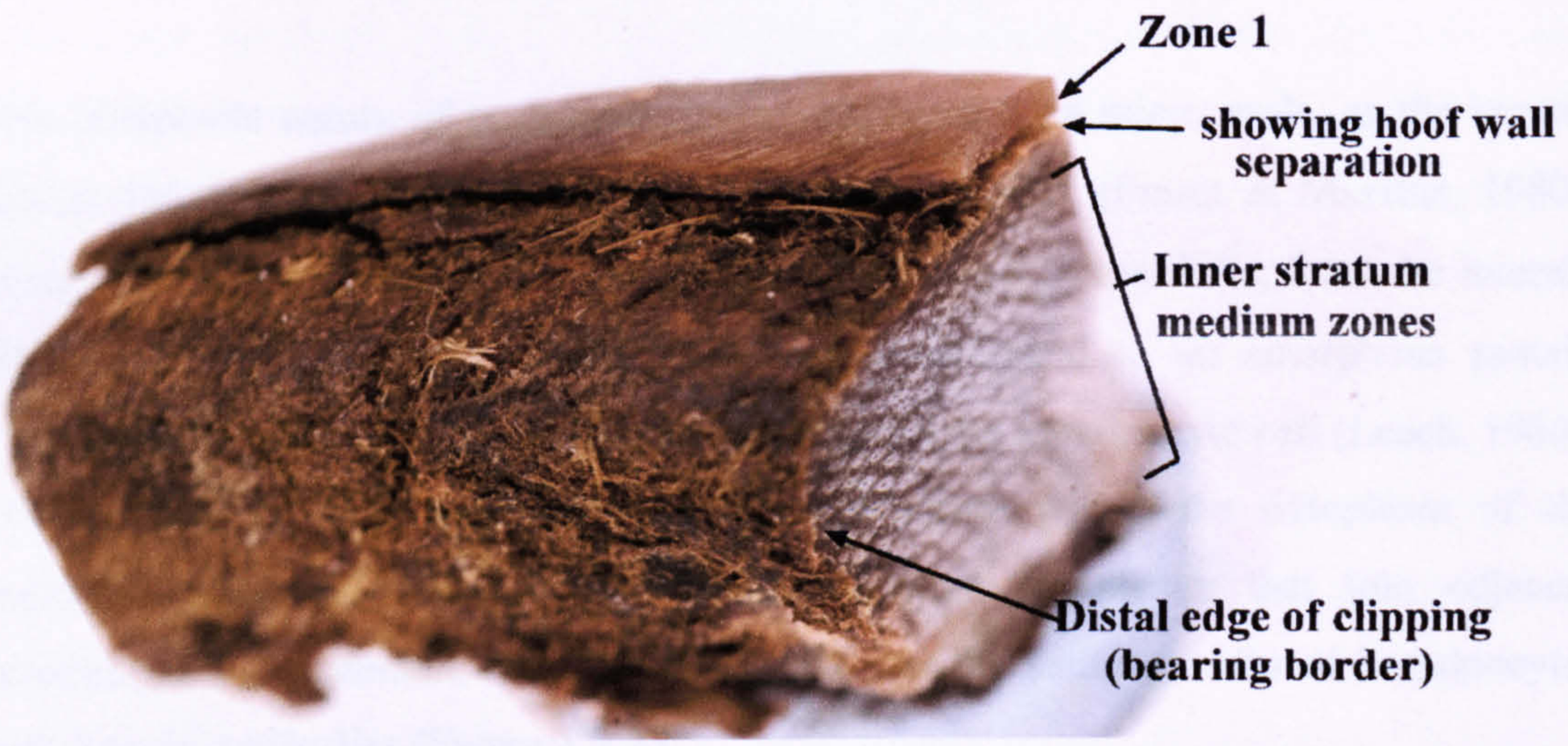


Figure 1.2.12 Hoof wall separation at zone 1 in the donkey (author's own photograph)

Reilly *et al.,* (1996) also believed that the tubules contributed to the normal function of the hoof by facilitating stress transfer and preventing crack propagation. They also went as far to say that damage to sensitive tissues was prevented by controlled delamination of damaged hoof wall. The *stratum medium* has been categorised into zones by a number of author's based on tubule type and density (see Section 1.2.12 for further details on tubule density and zonation). Personal observation of donkeys has revealed that delamination of the outer Zone 1 (Figure 1.2.12) does occur, but does not result in clinical signs of lameness. Thomason *et al.,* (1992) attributed the change in properties at the distal margin of the hoof to the hydration gradients within the hoof material.

Design complexity in both the tubular and intertubular components facilitates the control of crack growth (Kasapi & Gosline, 1999) and appears to increase fracture toughness, whilst the tubules themselves offer resistance against buckling (Kasapi & Gosline, 1998), similar to the effect of hollow fibres being present in a composite.

Vincent, (1990) defined a composite as being composed of stiff strong fibres, a discontinuous phase, within a relatively compliant matrix known as the continuous phase. In equid hoof the tubules of hoof horn can be described as the discontinuous phase and the intertubular horn is the continuous phase.

This composite nature of hoof horn is also displayed on a micro scale, as the keratin protein intermediate filaments are present within a matrix (Fraser & MacRae, 1980). Intermediate filaments (IF) of long slender alpha-helical microfibrils, form the internal skeleton of keratinocytes. These fibres are cross-linked to an amorphous protein interfibrillar matrix. The two phases completely fill the keratinocyte cell (Leach, 1980). These filaments form a three dimensional structure within the cytoplasm of the epithelial cells and terminate at the desmosomal complexes that join adjacent keratinocytes (Henderson, 2007). This can be seen by staining cultured keratinocytes with keratin antibodies (Figure 1.2.13).

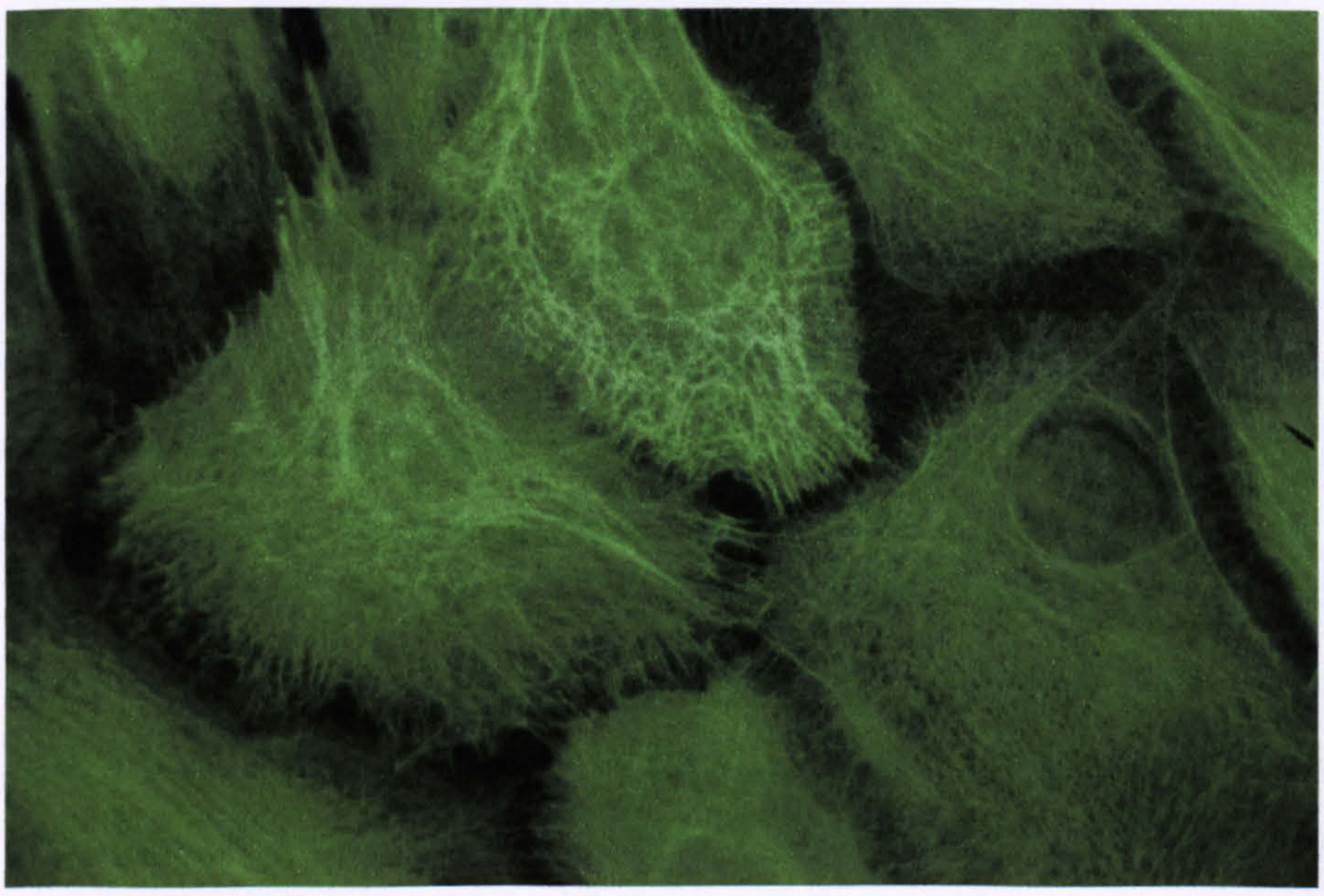


Figure 1.2.13 Cytoskeleton of Cultured Epidermal Cells (Bowden, in Henderson, 2007)

Primary keratinocytes treated with anti-keratin antibody and detected by immunofluorescence microscopy with FITC. The three-dimensional keratin IF network is clearly visible throughout the cells cytoplasm.

1.2.9 Keratinisation

The word keratin originates from the Greek Keros (Keros) meaning "horn" (Fraser *et al,* 1972). Keratins are polypeptides and are a family of proteins produced by keratinocytes which die in the terminal stage of keratinisation (Fraser & MacRae, 1980). In mammals, cytokeratins are softer epithelial keratins while hair keratins are harder. As certain skin cells differentiate and cornify, pre-keratin polypeptides are incorporated into intermediate filaments. Eventually, the nucleus and cytoplasmic organelles disappear, metabolism ceases, and cells undergo a programmed death as they become fully keratinized (Figure 1.2.14).

Keratinisation is the mechanism by which epidermal tissues are rendered tough and insoluble (Mercer, 1961). Keratins are the main constituent of structures that grow from the skin. Keratins can form α-helices and β-sheets. The α-keratin helix is present in the hair (including wool) and the horns, nails, claws and hooves of mammals. Keratin in the β-form produces sheet structures in the scales and claws of reptiles, as well as the feathers, beaks and claws of birds (Fraser & MacRae, 1980). Keratinous structures are necessary for protection from the environment, travel, search for food and the maintenance of the species (Leveque, 1994).

α-keratins are characterised by their fibrous nature and elastic properties. The mechanical strength and elasticity is derived from the organised systematic structure of the helix-type coils within the keratin fibres (Watt, 1980).

Not all keratins are identical, Grosenbaugh & Hood (1993) and Pellmann, Reese & Bragulla (1993) isolated keratins found in the equine hoof wall at the coronary band, the laminar epithelium, and the fully cornified hoof wall and characterised them with respect to molecular weight and immunoreactivity. All cytokeratin molecules contain central alpha helical segments and variable amino- and carboxyl-terminal domains; the difference in sizes between different keratins is due to variable end domains (Wattle, 1998).

The majority of research on keratins has been based on hair, wool and skin, through the cosmetics and textiles industries. Similarities have been shown to exist between the hoof and these other epidermal tissues (Mercer, 1961), so it is believed that the keratinisation processes for these structures are comparable with that for hoof horn.

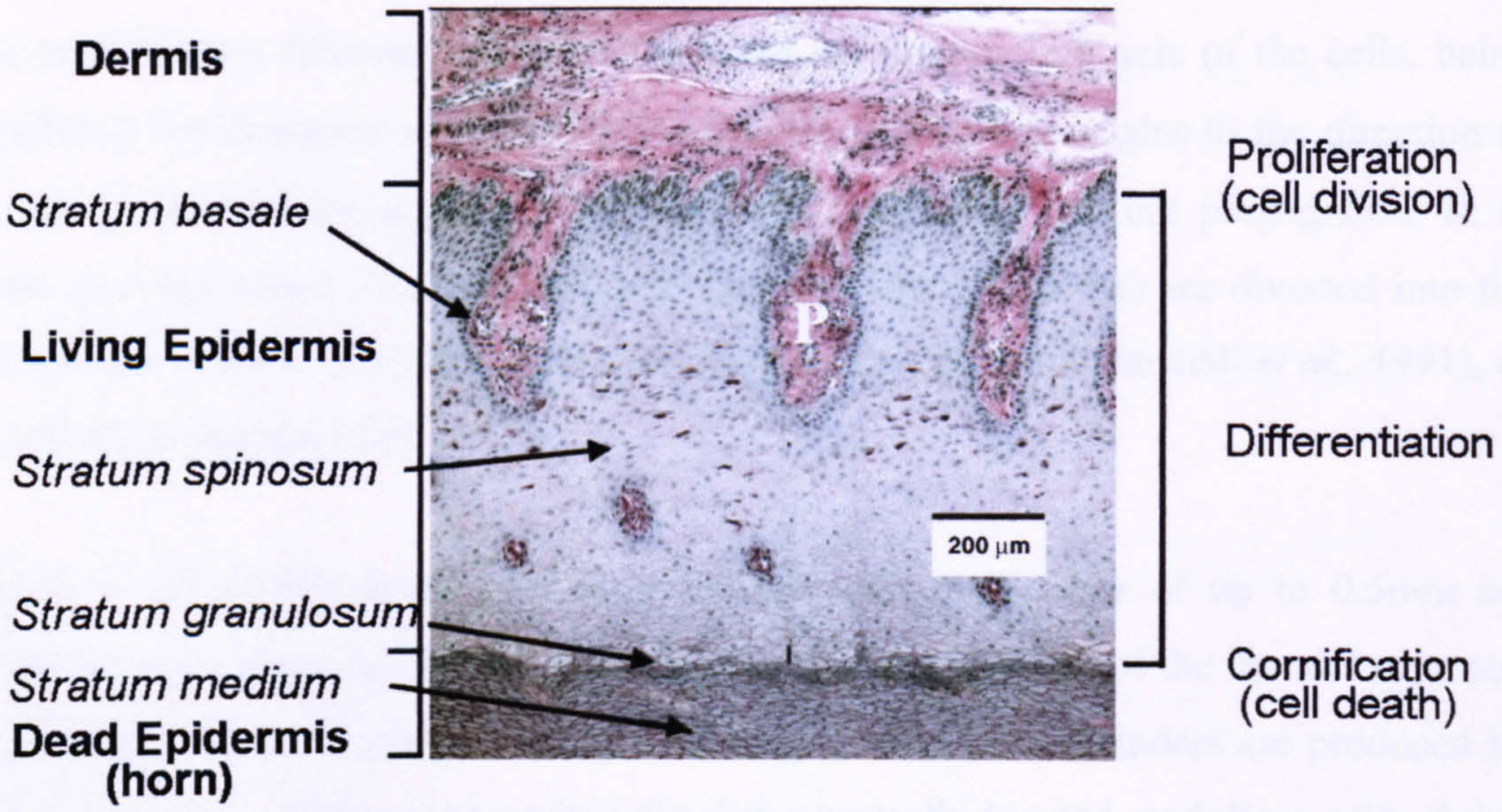


Figure 1.2.14 Heel region of bovine hoof (periodic acid schiff stain) showing dermis, dermal papillae (P), and cells undergoing differentiation, proliferation and cornification (adapted from Tomlinson *et al.*, 2004).

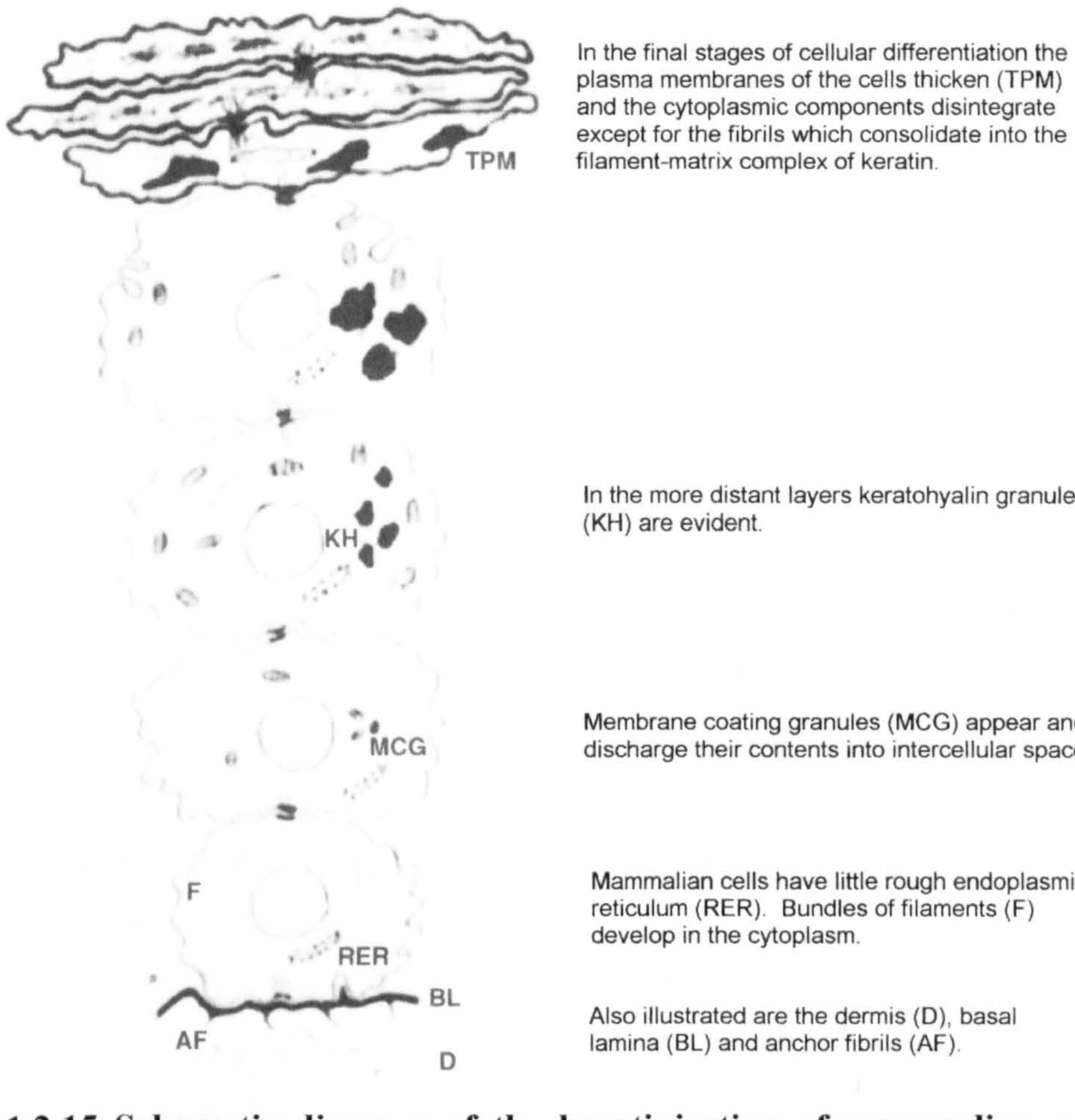
Within the living layer at the coronary corium, there are similar layers to those found in skin (Figure 1.2.14). The *stratum basale* is where proliferation of cells occurs prior to differentiation, which occurs in the *stratum spinosum*. Within the hoof there is an intermediary layer between the *stratum spinosum* and the *stratum medium*, known as the *stratum granulosum* (Bolliger, 1991).

The keratinised cells of the hoof wall coronary groove located beneath the coronary band (Figure 1.2.1) form numerous cone shaped holes or sockets. The tapering papillae (denoted by the P on Figure 1.2.14) of the coronary dermis fit tightly into the sockets

and, being highly vascular, supply nutrients and oxygen to maintain growth of the hoof wall throughout the life of the horse (Pollitt, 1995). Tubule cortex cells originate from the *stratum basale* lining the sides of the dermal papillae (Figure 1.2.1) and, once formed, these cells attach to the more fully keratinised intertubular horn cells, move distally within the *stratum spinosum* and progressively keratinise (Leach, 1980) (Figures 1.2.14 and 1.2.15).

The predominant filament orientation follows the longitudinal axis of the cells, being parallel to the direction of growth in the tubules and at right angles to the direction of growth in intertubular regions. This arrangement affects fracture propagation, in as much as longitudinal cracks (parallel to the direction of growth) are diverted into the intertubular regions, at right angles to the direction of growth (Marshall *et al.,* 1991), as described in Section 1.2.9.

Budras *et al.* (1998) found that horn tubules with a diameter of up to 0.5mm are produced in all segments of the equine hoof with the exception of the coronet segment, where laminar horn is produced. At their origin, solid horn cylinders are produced by epidermal cells. As they are pushed distal the centrally located medullary cells shrink markedly. Sturdier cortical cells remain intact, resulting in centrally empty horn tubules.



In the final stages of cellular differentiation the plasma membranes of the cells thicken (TPM) and the cytoplasmic components disintegrate except for the fibrils which consolidate into the filament-matrix complex of keratin.

In the more distant layers keratohyalin granules (KH) are evident.

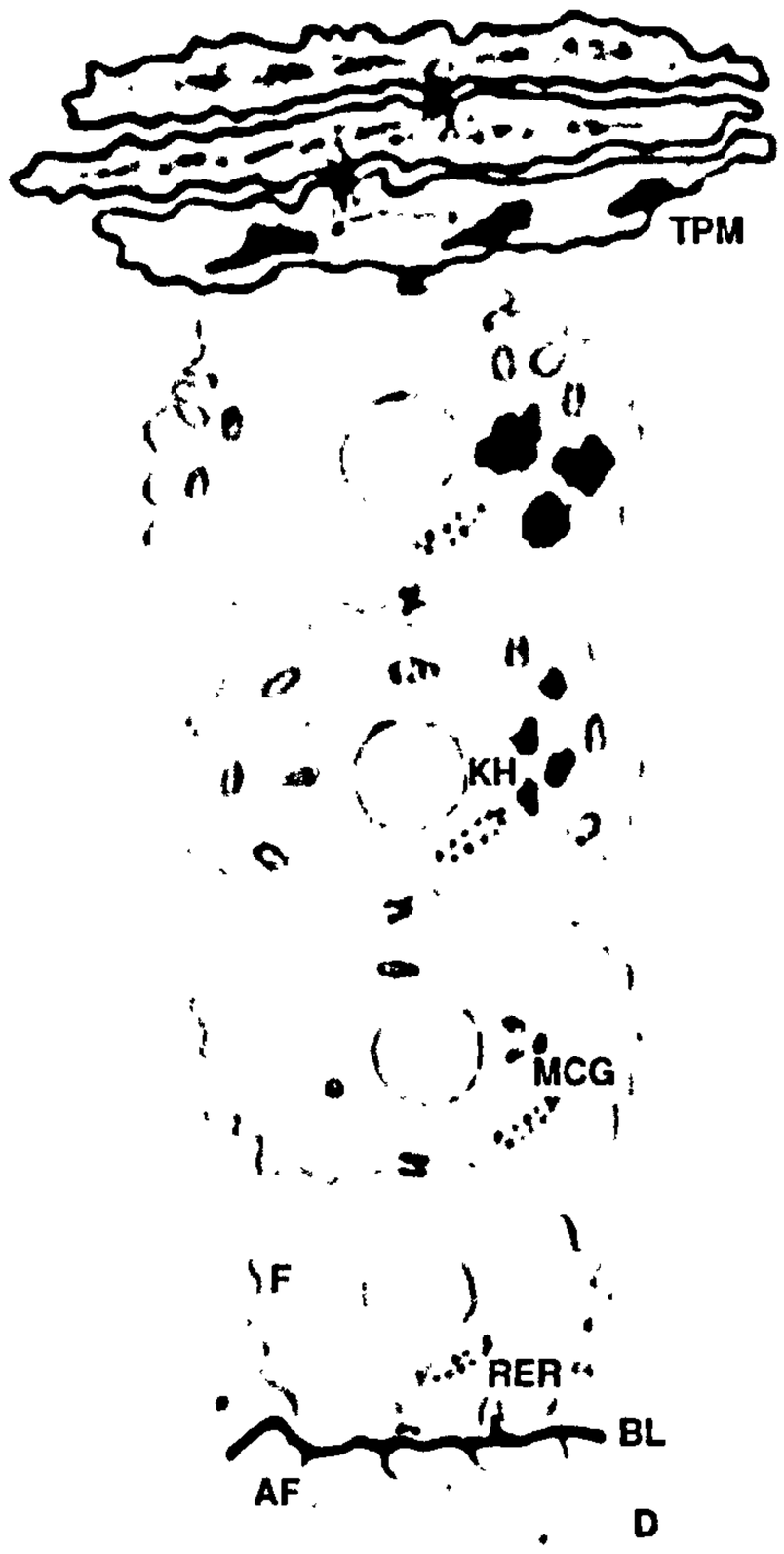
Membrane coating granules (MCG) appear and discharge their contents into intercellular spaces.

Mammalian cells have little rough endoplasmic reticulum (RER). Bundles of filaments (F) develop in the cytoplasm.

Also illustrated are the dermis (D), basal lamina (BL) and anchor fibrils (AF).

Figure 1.2.15 Schematic diagram of the keratinisation of mammalian epidermal cells (adapted from Tomlinson *et al.*, 2004)

An investigation into the growth of bovine hoof using carbon stable isotope ratios by Harrison *et al.,* (2007) revealed that the inner layers of the bovine hoof were younger than the outer layers. The offset in age of the hoof horn varied from 9.2 ± 1.8 days per mm at high hoof growth rates, to 14.0 ± 2.8 days per mm at low hoof growth rates. Horn is laid down at different times through the thickness of the hoof wall due to the arrangement of the dermal papillae which is very similar to that seen in equids (Figure 1.2.1). This is of importance when sampling hoof horn in zonal fractions, as the different zones of the hoof will have been created at different times in the animal's life and may therefore have been subjected to different dietary, environmental, or other physiological stresses.



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Cornification includes not only keratinisation (Figure 1.2.15), but also the production and cross-linking of cell envelope proteins and the formation of the intercellular lipid matrix (Grosenbaugh & Hood, 1993). The ultimate goal of cornification is a structure hard enough to withstand enormous forces and resilient enough to resist permanent deformation. Horse hoof is one of the toughest natural materials known. The hoof wall, composed of keratin, is rich in disulphide bonds. These give it strength, hardness and insolubility (Pollitt, 1998).

Hoof keratin cannot be regenerated or remodelled *in situ* because the keratinocytes from which it is derived die in the final stages of their differentiation (Grosenbaugh & Hood, 1993). Consequently, the material properties required for the function of the hoof horn are conferred through the structural design at the time of tissue deposition.

Cornification also includes the synthesis of the fundamental keratin polypeptides, forming intermediate filaments, which are then organised into a cytoskeleton within the cell by another group of proteins referred to as the intermediate filament associated proteins (IFAP) (Thomlinson *et al,* 2004). Cornification continues when the cell membrane is made rigid through the cross-linking of yet another group of proteins – the cell envelope (CE) proteins (Grosenbaugh & Hood, 1993).

Calcium-dependent transglutaminase enzymes are necessary to cross-link CE proteins to form hoof horn (Duren, 1998). Cysteine appears to be preferentially incorporated into the maturing cornified structure (the IFAP and CE proteins) and Grosenbaugh & Hood (1993) suggested it may be an essential component of the final cornification process. Once the cell envelope is formed (Figure 1.2.15), the precursor proteins become insoluble to even the harshest extraction techniques.

1.2.10 Lipids associated with cornification

As the final step in the cornification of the hoof wall, the keratinocytes secrete lipids into the extracellular space. When stained with acid haematin, the majority of the

cornified extracellular matrix of the hoof wall appears to contain phospholipids Grosenbaugh & Hood (1993). However, Kempson & Robb (1999) found that two of the horses (one a control and one a chronic laminitic horse with infected horn) which took part in their hoof care study shared a different pattern of hoof fatty acid composition to the other horses. This may be due to genetic differences in enzyme activity or dietary influences (discussed in section 2.2.4). Reilly *et al.*, (1998b) found that in Evening Primrose Oil Mixture (EPOM) supplemented animals, the periople showed significant differences in lipid content compared with controls, thereby providing direct evidence for a nutrient-hoof horn axis. Simmins (1985) also found that a biotin-deficient diet lead to decreased lipid fractions in the hoof horn of pigs during pregnancy and throughout lactation, and deduced that fat metabolism in pigs appears to be linked to dietary biotin intake.

1.2.11 Tubule density

The tubule density of hoof horn is typically defined by the number of tubule cavities per mm^2 (Leach, 1980; Reilly, 1999 and Hopegood, 2002). The tubule morphology is known to differ across the depth of the hoof wall. Kasapi & Gosline (1997) characterised five different tubule morphologies based on intermediate filament orientation of each cortical lamella.

The determination of tubule density provides a means of comparing basic anatomical features in hoof horn from different species. As well as macroscopic differences between the hoof structures of horses and donkeys, differences have been identified at microscopic level. Hifny & Misk (1983) also recognised that the hooves of donkeys differ from those of horses in respect of the size and density of horn 'tubes', although they did not provide any data in support of their statement.

Hoof function and the mechanical properties are believed to be influenced by tubule density as well as moisture content. Wilkens (1964) believed that the mechanical

strength of the hoof wall was dependent upon tubule density. Tubule density itself may provide some indication of the mechanical and structural properties of the hoof wall.

Kasapi & Gosline (1998) carried out tensile stress-strain curves for inner, middle and outer regions of fully hydrated equine hoof wall. The initial stiffness, which can be defined as the capacity to sustain loads without excessive deformation (Rivin, 1999), increased progressively from the inner to the outer region of the wall, although the shapes of the curves were similar beyond the 'yield' region. This corresponded with their data for tubule density across hoof wall depth.

Schummer *et al.*, (1981) hypothesised that tubule density may also be related to the resistance to wear but no supporting evidence was provided to demonstrate this claim. Fowler (1995) indicated that donkeys working in their natural environment do not generally need to be shod, which may be due to increased resistance to wear. Further investigation is required to ascertain whether there is indeed an increased resistance to wear in donkey hoof, and whether this can be attributed to morphological differences within the hoof horn.

Hoof hardness in other species, such as cattle and pigs, may be related to tubule density (Gunther *et al.*, 1983; Geyer & Tagwerker 1986), although mechanical tests were not carried out to substantiate these claims. Zoerb & Leach (1978) and Leach (1980) showed a positive relationship between modulus of elasticity and number of tubules. However, their tubule data was not expressed as the number of tubules per unit area. During an oral biotin supplementation trial using ponies, Reilly (1999) found that tubule density influenced flexural stiffness of hoof horn. Similarly, Hopegood (2002) analysed zonal moduli of elasticity in donkey hoof horn and found that the moduli generally decreased in a dorso-palmar direction, which is desirable as a low modulus is required where flexibility is needed. Flexural stiffness is intrinsically related to elastic modulus, although the elastic modulus is a property of the constituent material, in this case the hoof keratin, whereas the stiffness is a property of the solid body, in the case of the equid, the whole hoof.

1.2.12 Zonation in the *stratum medium*

According to Reilly *et al.*, (1996), the control of crack growth and stress transfer may be further facilitated by zonal tubule density variation. The highest tubule density occurs in the outermost layer and the density declines inwards forming a possible mechanism for smooth energy transfer from rigid outer wall to more plastic inner wall. The *stratum medium* has been categorised into zones by a number of author's based on tubule type and density. In horse hoof horn, Kasapi & Gosline (1998) divided the *stratum medium* into nine regions of approximate equal thickness, but these divisions did not take into account the tubule density or morphology. Leach (1980) found three distinct regions, based on visual identification of three tubule types. *Stratum medium* zones, based on tubule density at the midline dead centre sampling site, have been quantified for donkey (Hopegood, 2002) into three zones, and horse and pony hoof into four zones (Reilly *et al.*, 1996, 1998c).

Hopegood (2002) compared her results for donkey hoof horn with those of Reilly *et al.*, (1996 & 1998c) for pony and horse hoof horn, and found differences between the pony and horse hoof, which had similar tubule densities (Table 1.2.2) and zonal boundaries (Table 1.2.3), to the donkey hoof, which had lower tubule density, and different zonation boundaries. Both Reilly *et al.*, (1996 & 1998c) and Hopegood (2002) based their zonation of the *stratum medium* on changes in tubule density (Tables 1.2.2 and 1.2.3).

Table 1.2.2 Comparison of tubule density between species (tubules/mm^2) (adapted from Hopegood (2002) and extended)

	Donkey* (Tubules/mm^2)		Pony** (Tubules/mm^2)		Horse*** (Tubules/mm^2)	
	Zonal	Median	Zonal	Median	Zonal	Median
All Zones	10	10	16	16	16	16
Zone 1	>34	19	>27	27	>22	21
Zone 2	16-34	10	16-27	19	16-22	16
Zone 3	10-16	8	8-16	15	11-16	15
Zone 4	<10	9	<8	7	<11	9

* Hopegood (2002) ** Reilly *et al.*, (1996) *** Reilly *et al.*, (1998c)

Table 1.2.3 Comparison of zonal boundaries between species (% hoof wall depth) (adapted from Hopegood (2002) and extended)

	Donkey*	Pony**	Horse***
Zone 1-2	31	26	25
Zone 2-3	52	51	47
Zone 3-4	73	77	69

* Hopegood (2002) ** Reilly *et al.*, (1996) *** Reilly *et al.*, (1998c)

Differences in tubule morphometry and density may be seen in the four *stratum medium* zones when freshly cut sections of hoof are observed with the naked eye (Figures 1.2.16 and 1.2.17). Rapid recognition of the differences between the zones is especially important when processing zonal samples for moisture content analysis, as the hoof loses moisture at a rapid rate once it is exposed to the air following unwrapping. The main differences between the zones are explained and can be seen in Figures 1.2.16 and 1.2.17.

Zone 1 small oval tubules small amount of intertubular horn, high tubule density

Zone 2 combination of oval and round tubules, higher proportion of intertubular horn, and lower tubule density

Zone 3 large tubules higher proportion of intertublar horn than Zone 2, so lower tubule density

Zone 4 - larger tubules more intertubular horn lower tubule density

Figure 1.2.16 Donkey hoof wall zonal observations

Zone 1 Small oval tubules small amount of intertubular horn, high tubule density

Zone 2 small oval tubules, slightly more intertubular horn, lower tubule density

Zone 3 larger oval and round tubules, more intertubular horn, lower tubule density

Zone 4 large round tubules little room for intertubular horn, low tubule density

Figure 1.2.17 Pony hoof wall zonal observations

1.2.13 Tubule density and moisture content of hoof horn

Butler (1977), Vermunt & Greenhough (1995), Baillie & Fiford (1996) have linked moisture content to the tubules of hoof horn. Although Kasapi & Gosline (1998) found that moisture content was not significantly higher with the presence of tubules. Hopegood (2002) found a decrease in hydrated moisture content at higher tubule densities, with R^2 values of 64% and 84% for Zones 3 and 4 respectively. Differences at microscopic level affect the water content and transport through the hoof horn (Kasapi & Gosline, 1998). As the moisture content is known to affect the mechanical properties of the hoof (Douglas *et al.,* 1996), the importance of water to the hoof should not be underestimated. The possible interaction of tubules with moisture content was discussed by Hopegood (2002) who hypothesised that tubules may play a greater role where there is a limited amount of water present, for example when the hoof horn is equilibriated at 75% RH. Further study is required, using relative humidity atmospheres, to fully ascertain the significance of environmental humidity on possible tubule/moisture interactions and the mechanical properties of the hoof.

1.3 Moisture content

1.3.1 *Stratum medium* moisture content

Water modulates the characteristics of many biological materials and so these materials are strongly influenced by their state of hydration (Jackson, 1992). Keratin is classified as a fibrous composite of relatively water-impenetrable microfibrils embedded in a water-penetrable matrix (Fraser *et al.,* 1972). The amount of water within hoof horn affects its functional mechanical properties. Indeed, Bertram and Gosline (1987) found that the stiffness of hoof-wall was influenced by water content to a greater degree than was the stiffness of other mammalian hard keratins.

The function of water in biological systems has been under investigation for over a century (Kuntz & Kauzmann, 1974). A review of the literature has yielded little quantitative information on the moisture content of the *stratum medium* of donkey, and no information on the moisture content of the *stratum medium* of mule hoof horn. Moisture content can be quantified, and has been reported previously by a number of author's for the full hoof wall depth of horse hoof horn, and by Hopegood (2002) for donkey hoof horn. Their findings are summarised in Table 1.3.1.

Moisture content analysis is generally derived by the determination of mass lost following dehydration, which has in the past involved a variety of gravimetric methods with measurements over differing periods of time. Methods previously used include oven drying at different choices of temperature, but also drying at room temperature (Hopegood, 2002). This area of work has been variously reviewed, but protocols are often unclear and different methods of dehydration have been used. Until the work of Hopegood (2002), there appeared to be no standard method of moisture content analysis for hoof horn. Drying over phosphorous pentoxide (P_2O_5) was used by Speakman & Cooper (1936) and Goodings (1935) for the drying of wool prior to experimentation. Hopegood (2002) established drying over phosphorous pentoxide (P_2O_5) to be the most reliable and reproducible method of dehydration.

Table 1.3.1 Published values for horse and donkey hoof horn moisture content

Author	Year	Sole	Frog	Wall			¼s	Method
				Outer	Inner	HWD		
Fleming	1871	36.00	42.00	-	-	16.12	-	No method given
Zschokke	1885	35.40	47.90	-	-	28.50	-	No method given
Smith	1887	45.27	34.70	-	-	20.00	-	No method given
Benedetti	1948	-	-	-	-	36.33	-	Dried @ 90-100°c until equilibration
Miyaki *et al*	1974	33.70	38.70	-	-	27.10	-	Oven dry @ 105-110°c 10hrs
Leach	1980	-	-	20.00	27.60	-	-	60°c 5 days under vacuum
Naumann	1984	-	-	-	-	28.50	-	Oven dry @ 105-110°c 10hrs
Bertram & Gosline	1987	-	-	-	-	40.20	-	Maximal hydration, soaked in water and dried @ 80°c for 5 days
Kainer	1989	30.00	50.00	-	-	25.00	-	No method given
Pollitt	1995	-	-	15.00	32.00	-	-	No method given
Douglas *et al*	1996	-	-	27.90	35.50	-	32.50	Dried @ 103.5°c until constant mass
Spitzlei	1996	28.00	-	-	-	33.00	-	Maximal hydration, soaked in water and dried @ 105°c until equilibration
Kasapi & Gosline	1997	-	-	35.00	48.00	-	-	Maximal hydration, soaked in water & dried @ 100°c 5 days
Hinterhofer *et al*	1998	31.50	34.60	-	-	22.70	-	65% humidity 6 days
Reilly	1999	-	-	-	-	21.14	-	Air Dried
Reilly	1999	-	-	-	-	24.34	-	Fully hydrated then air dried
Hopegood	2002	-	-	27.5	38.5	-	-	(Donkey horn) P_2O_5
Hopegood *et al*	2002	-	-	-	-	33.42	-	(Donkey horn) P_2O_5

1.3.2 Importance of moisture content in hoof horn

Hoof function is facilitated primarily by the anatomical structure, although the natural regulation of horn moisture content is of utmost importance, as it has a close

relationship to the biomechanical properties of hoof horn (Butler, 1992, Douglas *et al.*, 1996).

The concept that moisture affects hoof integrity was recognised by Vegitus in 385BC. He was cited by Wildenstein (1995) as noting that "the circumstances of standing in a very clean stable without manure or moisture, with stalls having oaken flooring, strengthens the hooves".

Lungwitz (1891) commented that excessive dryness caused reduced deformation of the hoof during weight bearing. Indeed, Smith (1921) and Lambert (1966) believed that correct hoof function was determined almost completely by moisture content. Lambert (1966) proposed that the spring-like action of the wall was due to the interaction between the dry outer wall and the moist inner wall. He also demonstrated that excess moisture caused the hoof to lose its ability to hold its size and shape under stress and through further studies in 1968, demonstrated that loss of moisture caused immediate contraction of clippings when removed from the hoof.

1.3.3 The influence of moisture content on the mechanical properties of hoof horn

An inverse relationship has been found between the moisture content of hoof horn and its stiffness or its ability to resist deformation (Bertram & Gosline, 1987; Hinterhofer *et al.*, 1998; Kasapi & Gosline, 1997; Collins *et al.*, 1998). The dorso-palmar decrease in stiffness across the hoof wall is associated with an increase in water content in the same direction (Leach, 1980; Kasapi & Gosline, 1997). *In vitro* experiments have demonstrated that decreased fracture resistance is associated with increased hoof horn hydration (Bertram & Gosline, 1987; Kasapi & Gosline, 1997). Mechanical testing carried out by Naumann (1984) showed that resistance to indentation was decreased with increased moisture content. Baillie *et al.*, (2000) also found that the stiffness of bovine hoof horn reduced by between 5-20% with increasing moisture content (Figure 1.3.1). Their results for bovine hoof horn, between 250 MPa and 8000 MPa for 0-37% moisture content, were broadly in agreement with those of Bertram and Gosline (1987)

for horse hoof horn, between 410MPa and 14,600 MPa at 100% and 0% relative humidity.

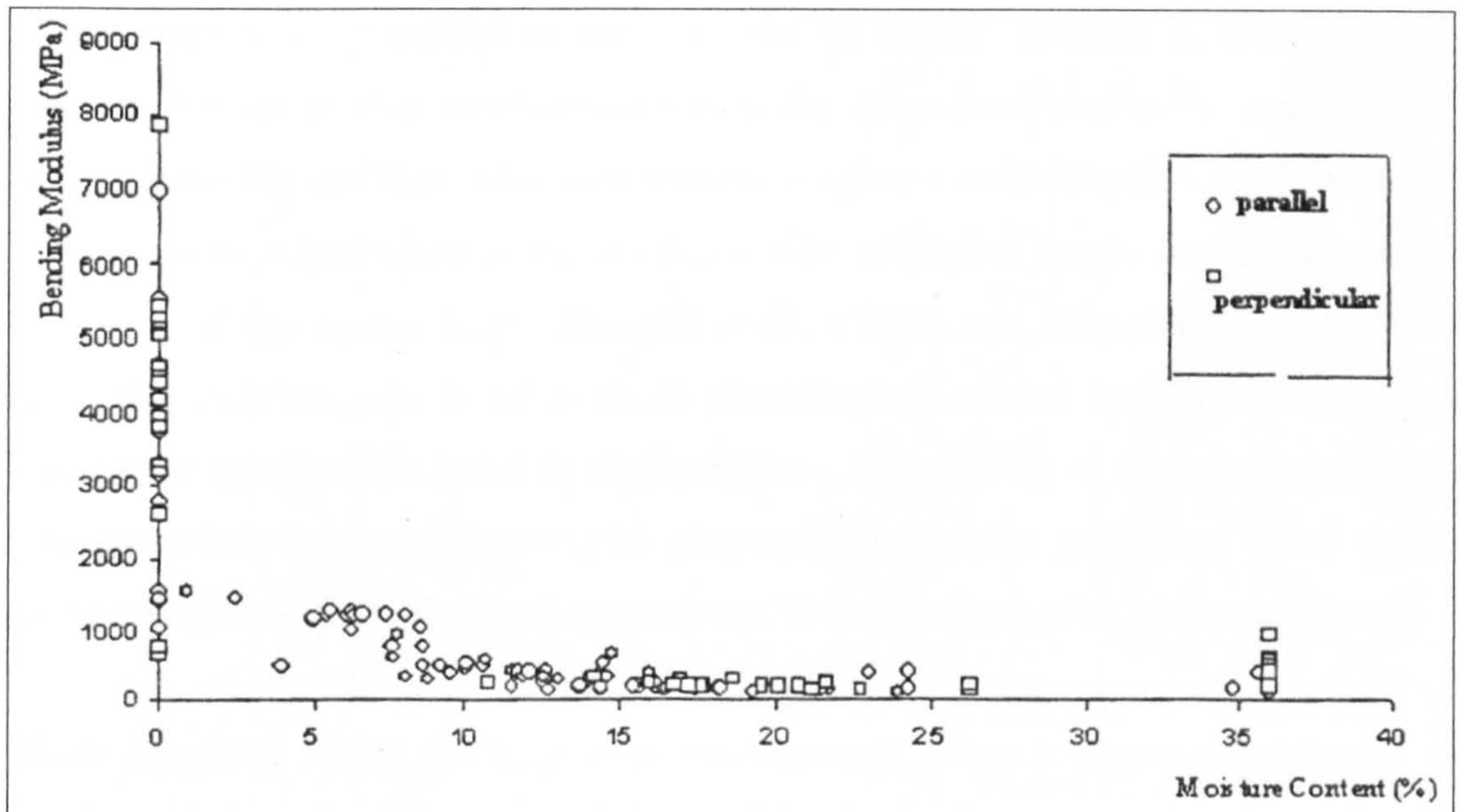


Fig. 15: Bending modulus versus moisture content for all data.

Figure 1.3.1 Bending modulus versus moisture content of bovine hoof (Baillie *et al.*, 2000)

Hopegood (2002) established that the mechanical properties of donkey hoof horn varied with hydration levels, and recorded a decrease in modulus of elasticity with an increase in hydration. Fresh hoof horn mean moduli for donkey was 177 MPa (43% regain moisture content), whereas the mean moduli for hydrated donkey hoof horn was 138 MPa (56% regain moisture content). These low moduli values are in broad agreement with the findings of Baillie *et al.,* (2000) for bovine hoof horn at moisture contents between 35-37%.

Ley *et al.,* (1998) did not find any correlation between percentage moisture content and tensile strength in horse hoof horn. This may have been due to the pooling of data from toe and heel regions for moisture content, but only the toe samples were used for strength testing. Zschokke (1885) found that moisture content varied between front and hind hooves.

It is imperative that the moisture content of samples is assessed at the same time as mechanical testing is carried out (Douglas *et al.*, 1996), so that accurate interpretations of the mechanical properties of the horn can be made. Bertram & Gosline (1987) deduced that the *in vivo* moisture content of the horse hoof was in the range 65-83% relative humidity and that maximum fracture toughness existed at this level, although, care has to be taken when using *in vitro* results to predict the *in vivo* biomechanical properties of the equine hoof. Douglas *et al.*, (1996) and Hinterhofer *et al.*, (1998) agree that only samples tested at these physiological relative humidity and moisture levels truly represent the actual *in vivo* condition. It is difficult to accurately replicate *in vivo* conditions in the laboratory, to date researchers have attempted to do this by ascertaining what RH% the hoof material equilibrates at fresh hoof moisture content.

Water contained within the keratinous structure may act as a plasticizer, whereby the water enables greater movement of the hoof keratin molecules as it has been found to plasticize human *stratum corneum* (Van Duzee, 1978). An increase in the water content results in changes to the hoof horn, altering it from being hard and brittle to soft and pliable (Vincent, 1990); thereby increasing the viscoelasticity of the hoof horn. This occurs as the water forces the polymer chains of the keratin apart (Watt, 1980), making them slide more easily over one another (Ashby & Jones, 1996).

1.3.4 The role of moisture content in hoof horn quality

A number of author's have proposed that hoof moisture content makes a large contribution to the "quality" of hoof horn (Leach, 1980; Kempson, 1990 and Budras *et al.*, 1998), but do not quantify exactly what moisture content is required for optimal hoof function. Naumann (1984) proposed that reduced hoof quality and brittleness would result if the moisture content of hoof horn was below a certain level, but did not provide any figures in support of his theory. Similarly, Budras *et al.*, (1998) suggested an "optimised" water content was favourable for horn "quality" but did not provide any information on what the definitive water content should be.

In fact a definition of "quality" itself is not explicit in the literature and "good quality" hooves must be inferred as those that have the appearance of being dense and smooth, and resist deformation and failure under mechanical testing (Zenker *et al.*, 1995), whereas poor quality hooves are those having brittle, cracked horn (Kempson, 1998).

1.3.5 Environmental factors influencing moisture content of hoof horn

It is known that the equine foot is rapidly responsive to both internal and external factors, it can change its shape and composition quickly (Moyer, 1989). Therefore, the factors affecting the individual horse are very important when analysing hoof quality, especially an appreciation of breed, husbandry conditions, and the sport type. When these factors are analysed, they could yield important information which may contribute to an explanation of the incidence of foot problems or lameness in individual horses.

Lack of understanding of the natural state and function of the hoof is one of the main problems when treating lameness (Emery *et al.*, 1977). It is important to try and simulate the environment in which horses evolved, in order to provide the best husbandry conditions. This concept is true particularly with regard to feet, one of the most specialised portions of equine anatomy.

The environment plays a major role in the genesis of foot damage and the best possible care can be provided through manipulation of this environment. The time of year and the surface can dramatically change the feet as well as the shoes. Horses that are subjected to wet grass in the morning and hot dry conditions later in the day often develop weak hoof walls and lose shoes easily (Moyer, 1989). Adverse environmental factors can negatively influence the outcome of some foot problems. The effect of the environment on the moisture content of the hoof horn can be studied in the laboratory by placing samples in controlled chambers and manipulating the atmospheric Relative Humidity (RH%) while maintaining a constant temperature. Data from the water

sorption and desorption by the hoof can then be plotted to produce an isotherm (see Section 1.3.7).

Budras & Schiel (1996) stated that stable hygiene and hoof care are of crucial importance for maintenance of hoof integrity. A study by Kempson (1987) demonstrated that bacterial invasion of horn is common in hoof defect cases, although she also found that the type of bedding was irrelevant, as long as it was clean and dry (Kempson, 1994).

A wide variety of commercially available products are imputed to provide the horse with a healthier, more functional hoof wall. Many of these products claim to prevent loss of the hoof wall moisture as well as provide moisture to the hoof wall. By applying these products to the hoof wall, horsemen hope to improve the quality of the hoof wall and its ability to withstand the forces placed on it during the wide variety of activities that are undertaken by the equine athlete today (Robertson *et al.*, 1996). Kempson (1994) emphasised that if the horn is already of poor quality acetone, hoof oils and fat solvents will create further damage.

It is questionable whether the artificial retention of excessive moisture by the application of a commercial hoof product is a good thing in the equine hoof, especially if the feet are over hydrated already, as excess moisture will then be locked within the hoof structure and this could lead to impaired hoof function. For instance, Baillie *et al.*, (2000) and Bertram and Gosline (1987) found that the stiffness of hoof horn became lower with increased moisture content, and so locking surplus moisture within the hoof wall could lead to changes in the material properties of the wall. Additionally, it is possible that a topically applied product may be altering wall components other than water content (Robertson *et al.*, 1996).

1.3.6 Effect of environmental relative humidity on the moisture content of hoof horn

Relative Humidity conditions can be produced in controlled chambers and this approach has been utilised in the laboratory to equilibrate natural materials prior to investigations of material properties. Materials studied in this way include skin (Blank, 1952) and human *stratum corneum* (Wildnauer *et al.*, 1971 and Van Duzee, 1978). One of the most common methods used to produce different relative humidity environments is to maintain a constant temperature, while using various saturated salt solutions (Rockland, 1960). The samples are then equilibrated in the environment created in the chamber by the salt solution. The sample is taken to be equilibrated when its mass remains constant over time.

In previous studies where horse hoof horn has been equilibrated over saturated salt solutions, the samples have been used for a variety of tests and investigations, but the reasoning for the equilibration and its relevance to their studies has not been given (Zenker *et al.*, 1995; Hinterhofer *et al.*, 1998).

As the hoof is greatly influenced by its water content, it is of particular importance that environmental conditions are standardised for mechanical testing. Kasapi & Gosline (1997) used a relative humidity environment of 97% to equilibrate samples prior to tensile testing, and the same author's in 1998 stored horse hoof horn samples over 33% and 75% relative humidity prior to mechanical testing. Hopegood (2002) also stored donkey hoof horn samples at 75% RH for 10 days prior to three point bending testing. The 75% RH is within Bertram & Gosline's (1987) predicted *in vivo* moisture content of horse hoof which was obtained within the relative humidity range of 65-83%. It could, therefore, be feasibly deduced that the *in vitro* hoof moduli obtained by Hopegood (2002) and Kasapi & Gosline (1997) were similar to those of *in vivo* conditions.

Hopegood (2002) also produced a sorption desorption isotherm for donkey hoof horn for full hoof wall depth samples, but no information is available for the hoof zones of the *stratum medium* and how they contribute to the full hoof wall depth isotherm.

1.4 Bound and free water content

Water exists in a number of states in biological materials. By establishing the total water content and then analysing the amount of 'freezable' water in a material, it is possible to determine the 'bound and 'free' water content of that material.

$$\text{Bound Water} = \text{Total Water} - \text{Free Water}$$

A number of author's have defined bound (unfreezable) and free (or freezable) water. Furuse *et al.*, (1984) stated that bound water encompasses the structural water of the cell wall and of various biological substrates of high molecular weight, such as proteins and polypeptides.

Franks (1984) defined bound water as including one or more of the following categories:

- A small number of water molecules may be found internally bound at protein pockets. These water molecules are an integral part of the protein itself.
- A larger percentage of the water molecules are associated with charged and polar surface sites on a protein.
- Hydration shell regions surrounding the proteins, which is of a variable extent.

Lundgren *et al.*, (1986) defined bulk water as that which is not associated with a protein or its variable hydration shell region. Bulk water is that which freezes at approximately 0°C, while bound water does not freeze. Differential scanning calorimetry is commonly used to establish the bound and free water fractions contained within biological materials such as *stratum corneum* (Inoue *et al.*, 1986; Imokawa *et al.*, 1991), and eye

lenses (Bettelheim *et al.*, 1982; Nunnari *et al.*, 1986; Popdimitrova & Bettelheim, 1989).

Bound water can also be attached to lipids. Inoue *et al.*, (1986) found 20-30% of the water in *stratum corneum* is bound and reacts strongly with proteins or lipids in the *stratum corneum*. They found that the 70-80% free water solubilised the water soluble components, such as amino acids, in the *stratum corneum*.

Imokawa *et al.*, (1991) also found that water could be bound to keratin fibres in the *stratum corneum*. They estimated that 20% of the bound water in the *stratum corneum* was bound to keratin, and *stratum corneum* lipids were capable of binding their own weight in bound water. In their study 0.133mg of the *stratum corneum* lipids could hold 0.13mg of the bound water.

There has been no research into the bound and free water fractions contained within equid hoof horn. Miles *et al.*, (1994) did use differential scanning calorimetry to look at denaturation of tendon degeneration in the horse, but the bound water content was not ascertained.

If the bound and free water fractions can be ascertained throughout the hoof wall depth, it may give some indication as to the lability of the water contained within the hoof, both at the lamellar junction, and at the external surface of the hoof. This baseline data could then be used to establish the effects of disease on hoof bound/free water fractions, or to assess the efficacy of topical ointments in maintaining a hoof moisture balance.

1.5 Hoof growth

1.5.1 Determination of hoof growth and growth rate

Reilly *et al.*, (1998a and 1998b) have already expressed the distinctions to be made between hoof growth and hoof growth rate. While hoof growth is an observation measured in cm or mm, it gives an indication of the amount of hoof produced over a given period (Graham *et al.*, 1994). The hoof material that is produced within that period can be used to calculate a growth rate. This involves recording growth over a given period and then dividing the time span to produce growth rate with units of cm/ month or mm/day (Josseck *et al.*, 1995). By recording the hoof wall height, the growth rate can also be used to calculate the renewal time (Geyer & Schultz, 1994). This is the time taken for the hoof capsule to be completely replaced by new growth, usually measured in days or months.

A review of the literature has revealed numerous studies concerning hoof growth in horses and ponies, but there have been no published studies about the hoof growth rates of donkeys and mules.

1.5.2 Growth rate of equid hoof horn

Kainer (1989) stated that the hoof wall grows distally at a rate of 8-10mm per month in the horse, which is the equivalent of 0.26-0.32mm/day. Various growth rates have been recorded for horses and ponies, according to breed, age, physiological circumstances and feeding (Table 1.5.1). At the growth rate stated by Kainer (1989), replacement of the wall at the toe would require approximately 12 months, at the quarters 6-8 months at the heels 4-5 months.

1.5.3 Circumferential growth of equid hoof horn

Whether growth of horn at different radial sites around the horse hoof capsule takes place at different rates is unclear; Kainer (1989) suggest that this growth is even, radially, around the coronary band, whereas Josseck *et al.*, (1995) found differences in growth rate between midline, lateral sidewall and palmar/plantar sites. The midline dead centre (MDC) is clearly defined and gives precision of measurement, enabling like for like comparisons to take place. It is unclear from the literature where the toes, quarters and heels measurement sites are located. Frackowiak & Komosa (2006) described 'collateral part-two' and 'movable part-two' as well as 'dorsal' measurement sites for their study into the dynamics of hoof growth of Konik horses. There was no diagram illustrating exactly where these locations were on the hoof, so it is unclear whether these measurement sites are comparable to the quarter, heel and toe measurement sites, which might be suggested from the descriptions.

Table 1.5.1 Equine hoof growth rates (mm/day)

Author	Animal Treatment Measurement Location	Growth Rate (mm/day)
Knezevic (1959)	Not Stated	0.29
Lungwitz & Adams (1966)	Not Stated	0.27
Butler & Hintz (1977) (ponies 8 months old)	Fed Ad Lib Limited Feed Not stated	0.38 0.25
Shannon & Butler (1979)	2 yo Spring 2yo Autumn 3yo Spring 3yo Autumn 4yo + Spring 4yo + Autumn MDC	0.24 0.27 0.24 0.26 0.23 0.19
Buffa *et al.*, (1992)	Biotin Control MDC	0.33 0.27
Geyer & Schultz (1994)	Shires Warmbloods Icelandics MDC	0.21-0.39 0.18 0.14-0.18
Graham *et al.* (1994)	Yearlings MDC	0.43
Josseck, Zenker & Geyer (1995)	Biotin Control Mean of dorsal/lateral/quarter	0.25 0.25
Reilly *et al.*, (1998a)	Biotin Control MDC	0.19 0.16
Reilly *et al.*, (1998b)	Evening Primrose Control MDC	0.18 0.21
Frackowiak & Komosa (2006)	Juvenille mares Spring Juvenille mares Summer Juvenille mares Autumn Juvenille mares Winter Adult mares Spring Adult mares Summer Adult mares Autumn Adult mares Winter Stallions Spring Stallions Summer Stallions Autumn Stallions Winter	0.14 0.32 0.22 0.20 0.14 0.25 0.18 0.18 0.13 0.26 0.24 0.19

1.5.4 Comparison of growth rate between different species

Relatively little is known about the growth of donkey and mule hooves, and as mentioned previously, there were no published studies about the hoof growth rates of donkeys and mules found in the literature review. Despite this, there are situations both in the UK, and abroad, where donkeys and mules are kept together with horses, at the same location, under the same environmental conditions. The hooves of each species may respond differently to changes in environmental conditions. In order to establish baseline values for normal hoof growth, a controlled trial is required where all three species are kept in the same environmental conditions. Any diversity in hoof growth rates may have implications for the farriery and day to day hoof care of the various species.

1.5.5 Manipulation of hoof growth and growth rate

Manipulation of hoof horn growth rate through optimal nutrition and supplementation can have practical implications for veterinary treatment and farriery in terms of growing out a lesion, or for producing sufficient horn for nailing in to (Reilly *et al.*, 1998a). It may also be of great importance in the donkey following remedial trimming of rescue cases that have suffered from abnormal hoof growth, in order to produce a balanced hoof, with a straight hoof pastern axis.

Many of the donkeys that are taken in at the Donkey Sanctuary in Devon suffer from neglected hooves on arrival, with gross overgrowth and frequently cracked hooves, requiring remedial farriery. In the wild in their native arid environment, donkeys hooves are trimmed naturally by the harsh ground conditions and constant movement. In captivity in a temperate environment, conditions are radically different and hoof care is extremely important. If the donkey hoof growth rate can be increased this will improve the welfare and reduce the convalescence period for donkeys that are growing out cracks or lesions.

1.5.6 Determination of hoof wear and wear rate

One study reporting the hoof wear rate of semi-feral Shetland sized ponies in Pennsylvania, USA (Florence & MacDonnell, 2006), was found in the literature. They analysed both growth and wear rates under conditions of self-trimming for the months of June-September, and daily wear rate varied from 0.52mm/day in July to 0.24mm/day in September. Florence & MacDonnell (2006) believed that natural hoof trimming coincided with a hardening substrate under-hoof that occurred with the changing climate and season.

No figures could be found for hoof wear rates throughout an entire calendar year in equid species in the literature. However, hoof wear rates have been studied in other species particularly cattle. It has been noted that increased wear rates are seen when cattle are kept on an abrasive surface such as concrete (Hahn *et al.,* 1986) and also that claw wear rates in cattle are higher in summer than in winter (Clarke & Rakes, 1982).

Hoof wear rates have not been studied in donkeys and mules, but also need to be considered, as the vast majority of donkeys and mules are unshod. If hoof wear exceeds hoof growth in unshod animals it can lead to bruising and clinical lameness (Mills *et al.,* 1986). If the animal is working, this has further implications for its usefulness as well as its health and wellbeing. For example lameness caused by excess wear and of sufficient duration or severity to prevent adequate feed intake in dairy cattle, may manifest itself as a drop in milk production, weight loss, or substandard weight gain (Mills *et al.,* 1986).

Any diversity in hoof wear rates may have implications for the day to day hoof care of the various species, and in cases where wear exceeds growth to the extent of clinical lameness, indicate a requirement for that animal to be shod. A positive growth:wear ratio must be maintained so that the animals do not get footsore or go lame.

1.6 Sampling hoof horn

Hoof horn samples can be obtained from hoof clippings which are taken at the time of routine farriery, from post mortem capsules or from hoof biopsies. Biopsies need to be taken by veterinary surgeons, and involve the administration of nerve blocks and painkillers. A full hoof wall depth biopsy would involve damage to the sensitive tissues of the foot and extensive post-operative care. Full hoof wall biopsies have been used by Pollitt & Daradka (2004) to investigate hoof wall repair processes.

Post mortem samples can also be used. They provide a full hoof capsule with a larger sampling area than clippings. Post mortem samples allow a more proximal sampling site, which avoids the sample being damaged mechanically, and affords more control over sampling. Post mortem samples were used by Reilly (1999) in an equine nutritional trial, in order to conduct full analyses of the effects of nutrition on the horn composition and structure, at various locations within the hoof wall.

Hoof clippings are the most readily available of hoof samples, and the least invasive. They can be collected at regular intervals, in conjunction with the regular farriery attention; this can vary from 6-10 week intervals dependent on the amount of hoof growth. Although hoof clippings are readily available, there are a few factors to be taken into consideration when using clippings as hoof wall samples. The bearing border is subject to damage by abrasion, both from rasping during farriery, and through natural hoof wear at the dorsal edge of the hoof wall during locomotion. Along with the age of the horn, and abrasion, the bearing border is also subject to environmental challenges such as contact with urea and faeces in bedding, as it is in direct contact with the ground surface (Budras, Schiel & Mulling, 1998). If ammonia from soiled bedding attacks hooves the unprotected horn can absorb urea, which will destructure hoof horn at the molecular level (Kung *et al.*, 1993).

1.7 Rationale for the programme of research

From the literature review it can be seen that there is limited quantitative information about the *stratum medium* of donkey hoof horn and no information on the *stratum medium* of mule hoof horn of animals maintained in a temperate climate. Nor have any direct comparisons with horses kept in the same environment been made. With respect to hoof moisture content, the assumption that donkeys are small horses cannot be refuted until a controlled trial is carried out, and evidence is presented to prove, or otherwise, that there are significant difference between the species.

Although bound and free water fractions have been investigated previously in sheep claw horn, there have been no comparable investigations in equid hoof horn. This parameter may provide useful information on the type of moisture contained within the hoof, and this may also have a bearing on the mechanical function or disease susceptibility of the hoof. Differential Scanning Calorimetry has been found to be useful in ascertaining the bound water content of *stratum corneum*, which has been located bound to lipids and keratins – both known to be constituents of *stratum medium* hoof horn.

It has often been hypothesised that the moisture contained in the external environment surrounding the equine hoof has a dramatic effect on the moisture content of the hoof horn itself, but only a few studies have been carried out which included equilibrating hoof horn at specific relative humidities. There are no data to illustrate the effect that the humidities have on the individual zones of the hoof wall *stratum medium*. If a sorption/desorption isotherm can be produced for each of the four hoof wall zones of the *stratum medium*, this may further our understanding of the interrelationships between the zones and the moisture gradient contained therein.

Previous studies on the hoof growth of horse and pony hoof horn have been carried out, but comparisons between them have been difficult due to differing methodologies, including sampling sites and method of measurement. No studies have been carried out

into the growth rate of mule or donkey hoof. A protocol needs to be established for the measurement of hoof horn growth, which can be used to ascertain quantitative values for the hoof growth rates of donkey, mule and pony hoof at the midline dead centre, quarters and heels. Hoof wear rates have not been studied in equids, but also need to be seriously considered, as the vast majority of donkeys and mules are unshod.

1.8 Objectives of the Thesis

For donkey, mule and pony hoof horn from a controlled experiment, where all three species were maintained in the same environmental conditions, under the same farriery regime, the objectives of this study were:

- To investigate the moisture content of full and partial hoof wall depth samples for donkey, mule and pony hoof horn at five defined sites around the circumference of the hoof capsule, over a period of 12 months.

- To compare the moisture content of full and partial hoof wall depth samples for donkey hoof horn with those for mule hoof horn and pony hoof horn, over a period of 12 months.

- To investigate the bound moisture content of full and partial hoof wall depth samples for donkey, mule and pony hoof horn at the midline dead centre sampling site on the front left hoof capsule, over a period of 12 months.

- To compare the bound moisture content of full and partial hoof wall depth samples for donkey with those for mule and pony hoof horn at the midline dead centre sampling site on the front left hoof capsule, over a period of 12 months.

- To investigate the hoof growth rate for donkey, mule and pony hoof horn at five defined sites around the circumference of the front left hoof capsule over a period of 12 months.

- To compare the hoof growth rate for donkey hoof horn at five defined sites around the circumference of the left front capsule with that for mule hoof horn and pony hoof horn at corresponding sites, over a period of 12 months.

- To investigate the wear rate for donkey, mule and pony hoof horn at five defined sites around the circumference of the front left hoof capsule over a period of 12 months.

- To compare the wear rate for donkey hoof horn at five defined sites around the circumference of the left front hoof capsule with that for mule hoof horn and pony hoof horn at corresponding sites, over a period of 12 months.

- To compare the hoof replacement interval for donkey, mule and pony hoof horn at five defined sites around the circumference of the front left hoof capsule.

- To investigate the inter-relationships between the quantitative results for moisture content, bound water content, and hoof growth rate for donkey, mule and pony hoof horn.

For donkey hoof horn, where the source and full husbandry history of the hoof horn was known:

- To establish the effect of different relative humidity environments on the moisture content of full and partial hoof wall depth samples of donkey hoof horn.

- To establish the effect of soaking full and partial hoof wall depth samples of donkey hoof horn in distilled water on the dimensions of the hoof horn.

CHAPTER 2 – EXPERIMENTAL METHODS

2.1 Introduction

Generic methods used for taking samples and sample processing are detailed in this Chapter. Specific methods used when analysing samples are described in relevant chapters.

2.1.1 Sampling hoof horn

A number of morbid donkey and horse hoof samples were used as part of this study to determine fully hydrated moisture content and differential scanning calorimetry analyses in preliminary studies. Hoof clippings from donkeys, mules and ponies were used for fresh moisture content determination and differential scanning analysis to determine bound water content in a year long trial based at the Donkey Sanctuary.

2.1.2 Sampling morbid hoof tissue

Whole feet were obtained from donkeys which were euthanised for reasons other than the present study. The feet were stored at approximately -23°C until sampling. Surface dirt was carefully washed off each foot using cold tap water, and excess surface water was then removed by blotting with a paper towel.

To mark the midline dead centre (MDC) sampling site (Reilly *et al.*, 1996) the hoof was inverted so that the sole surface was uppermost. The MDC was taken as being the line bisecting the sole in a direct line from the apex of the frog (Figure 2.1.1). This line was then continued up the dorsal face of the hoof wall, parallel to tubule alignment (Figure 2.1.2).

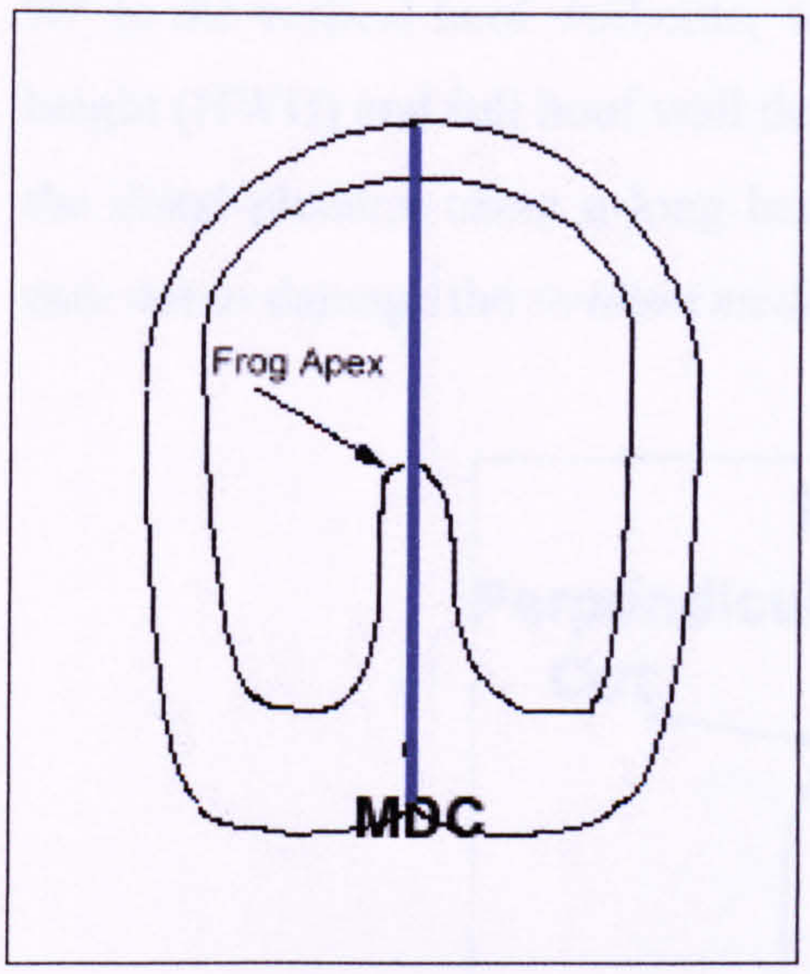


Figure 2.1.1 Ventral view of hoof to show MDC

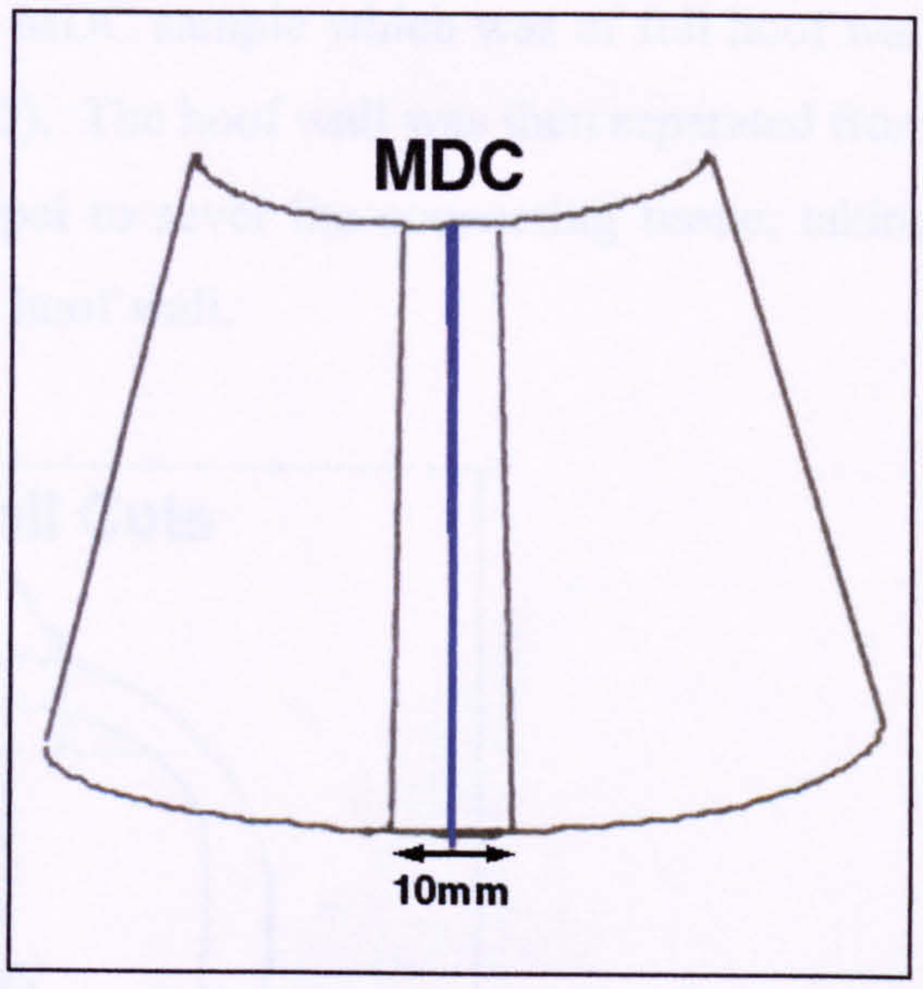


Figure 2.1.2 Dorsal view of hoof to show MDC

5mm measurements were then made either side of the marked MDC, to ensure a 10mm full wall width MDC section was taken (Figure 2.1.2). This was then sawn through the full wall depth to the distal phalanx using a hack saw (see Figure 2.1.3 for saw lines and Figure 2.1.5 for full hoof wall depth sample).

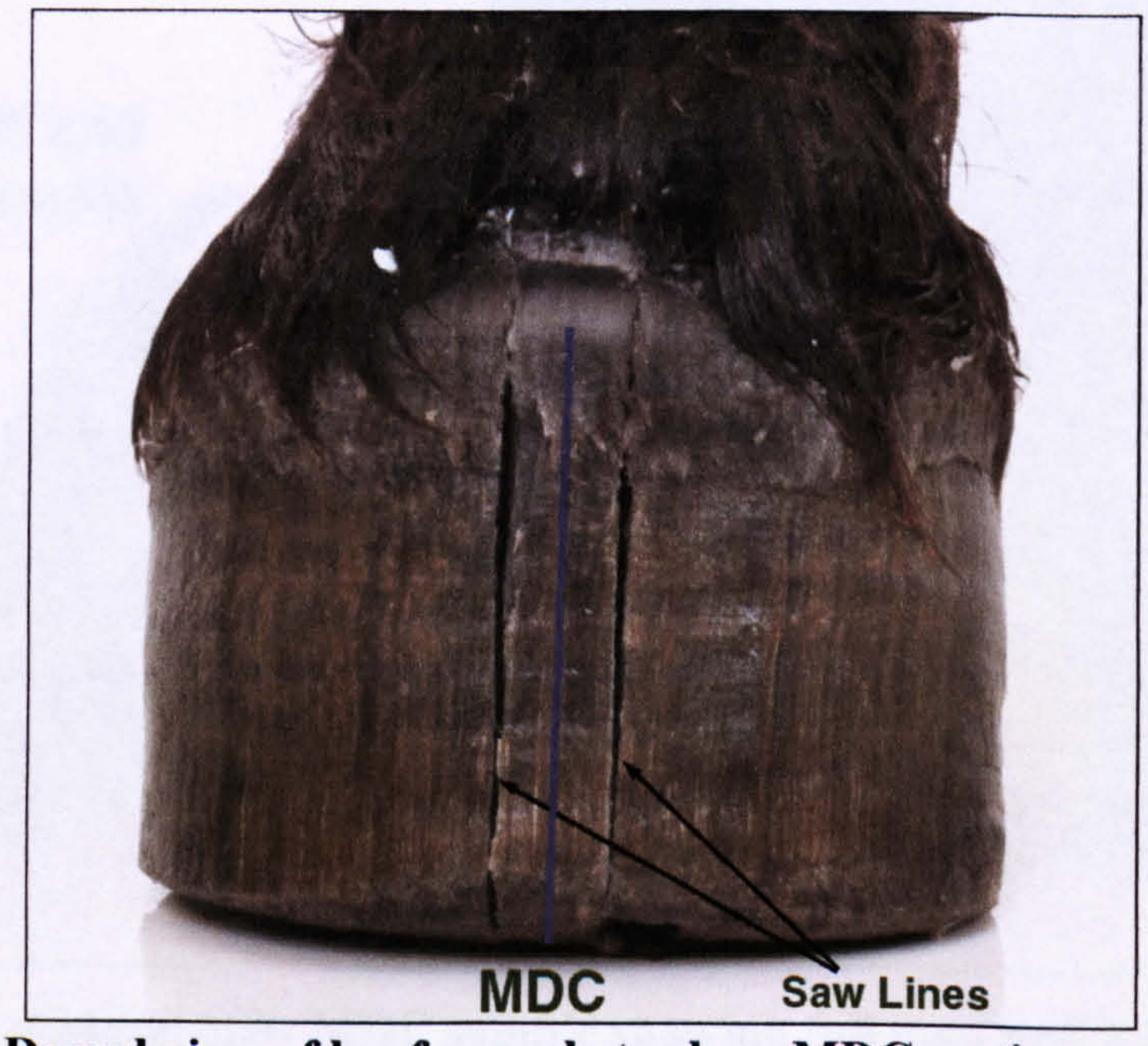


Figure 2.1.3 Dorsal view of hoof capsule to show MDC section

The hoof was then inverted again, and a perpendicular cut was made (Figure 2.1.4) at 90° to the vertical hoof wall cuts, to create a MDC sample which was of full hoof wall height (HWH) and full hoof wall depth (HWD). The hoof wall was then separated from the distal phalanx using a long handled scalpel to sever the connecting tissue, taking care not to damage the *stratum medium* of the hoof wall.

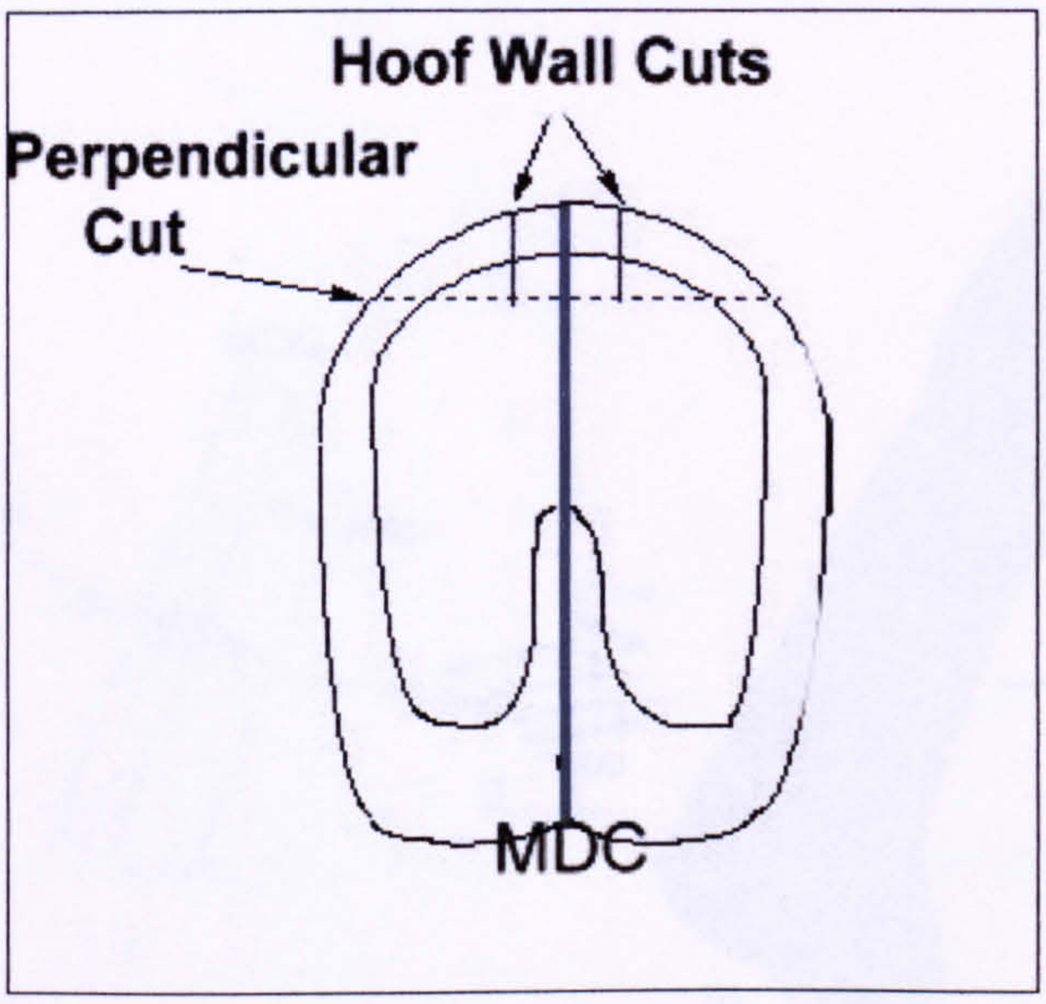
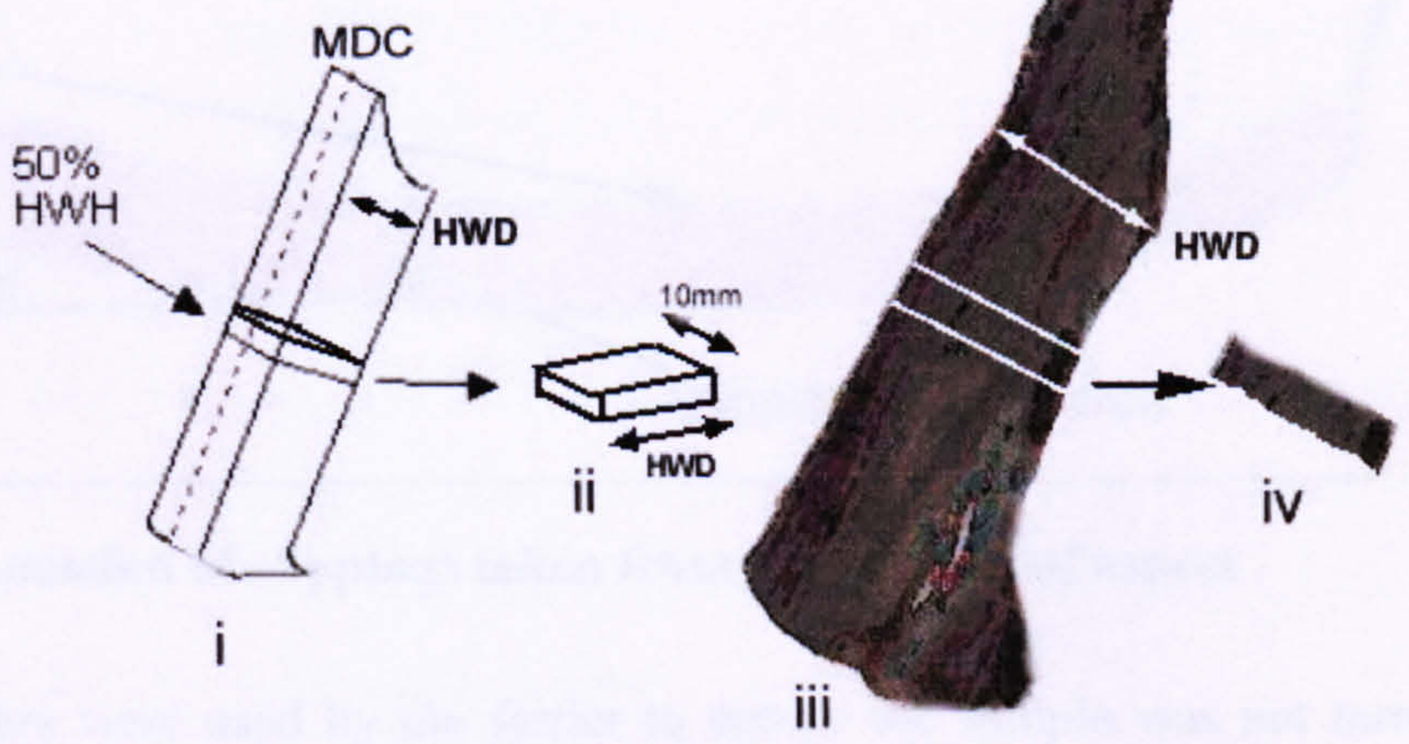


Figure 2.1.4 Perpendicular ventral cut

Figure 2.1.5 Lateral view MDC sample showing full hoof wall height (HWH) and depth sample

The resulting hoof horn section (Figure 2.1.5) was then sampled at 50% hoof wall height (50% HWH), to obtain a full hoof wall depth hoof horn wafer (ii and iv in Figure 2.1.6). The cuts were made 1mm above and 1mm below 50% HWH, to give a wafer that was 10mm wide, 2mm thick and extended for the full hoof wall depth which varied from animal to animal (Figure 2.1.6).



i and iii) Hoof wall MDC full hoof wall height and depth section
ii and iv) 2mm thick hoof wall wafer section taken at 50% hoof wall height

Figure 2.1.6 Lateral view MDC hoof section illustrating hoof wafer sampling

2.1.3 Hoof clipping sampling

Hoof clippings provide a non-destructive sampling opportunity, as they can be taken during regular hoof maintenance. Hoof clippings were obtained by the farrier from the distal aspect of the hoof of the equid (Figure 2.1.7). The clipping height depended on the growth and wear of the hoof between hoof maintenance intervals. On occasions, with some of the individuals, there was not sufficient hoof growth available to take a clipping sample without causing the animals discomfort and potentially causing them to be lame. If this was the case, a sample was not taken.

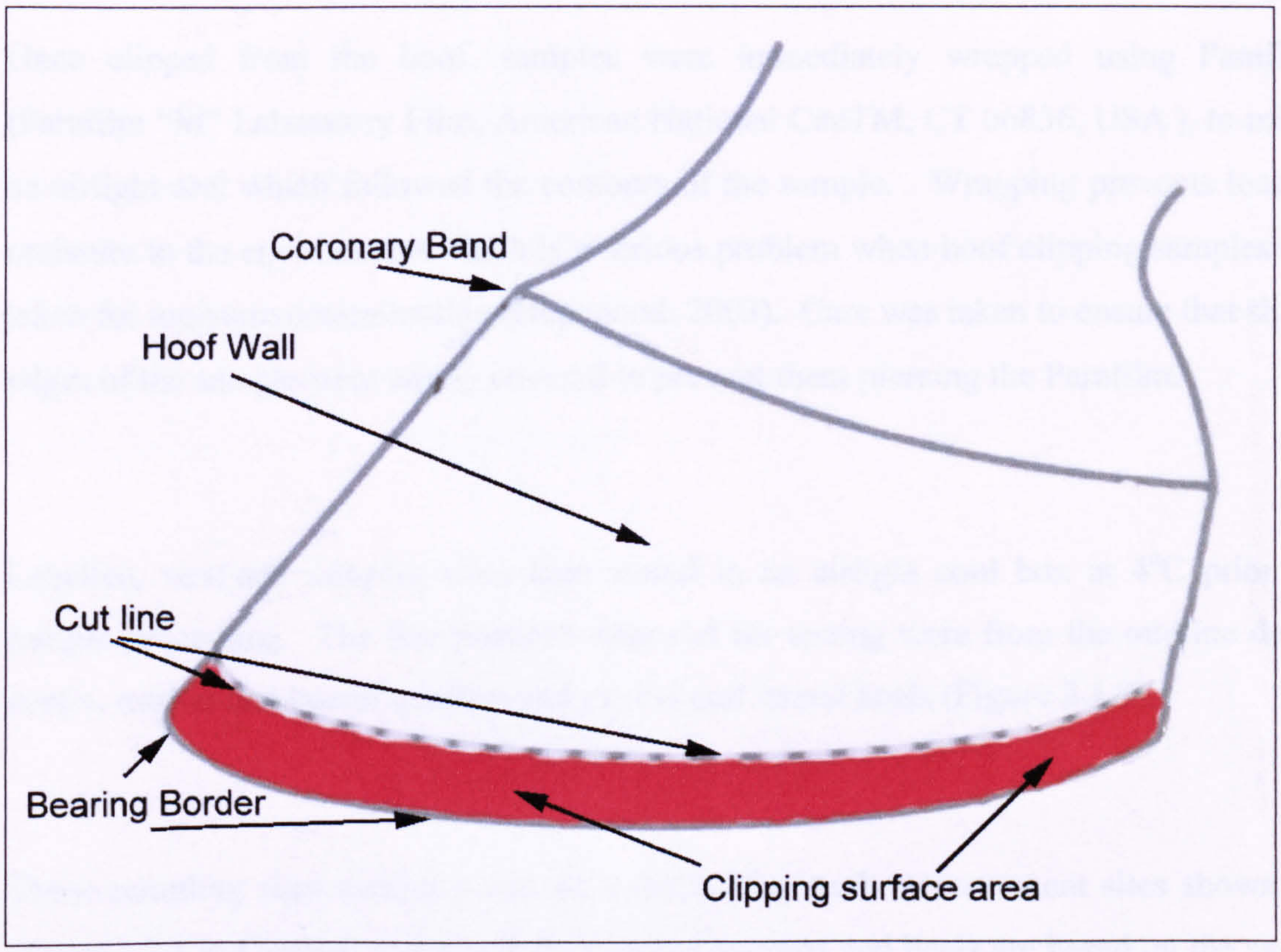


Figure 2.1.7 Location of clippings taken from the distal hoof aspect

Sharp hoof cutters were used by the farrier to ensure the sample was not torn during collection (Figure 2.1.8).

Figure 2.1.8 Farrier taking a clipping sample from a mule

Once clipped from the hoof, samples were immediately wrapped using Parafilm (Parafilm "M" Laboratory Film, American National CanTM, CT 06836, USA), to make an airtight seal which followed the contours of the sample. Wrapping prevents loss of moisture to the environment which is a serious problem when hoof clipping samples are taken for moisture determination (Hopegood, 2003). Care was taken to ensure that sharp edges of the sample were amply covered to prevent them piercing the Parafilm.

Labelled, wrapped samples were then stored in an airtight cool box at 4°C prior to sample processing. The five portions removed for testing were from the midline dead centre, medial and lateral quarters and medial and lateral heels (Figure 2.1.9).

These sampling sites corresponded with the hoof growth measurement sites shown in Figure 5.2.1 in Chapter 5, as the definitions of quarters and heels are based on the same measurements; from the point of the frog, and 50% of the distance between the point of the frog and the toe at the midline dead centre.

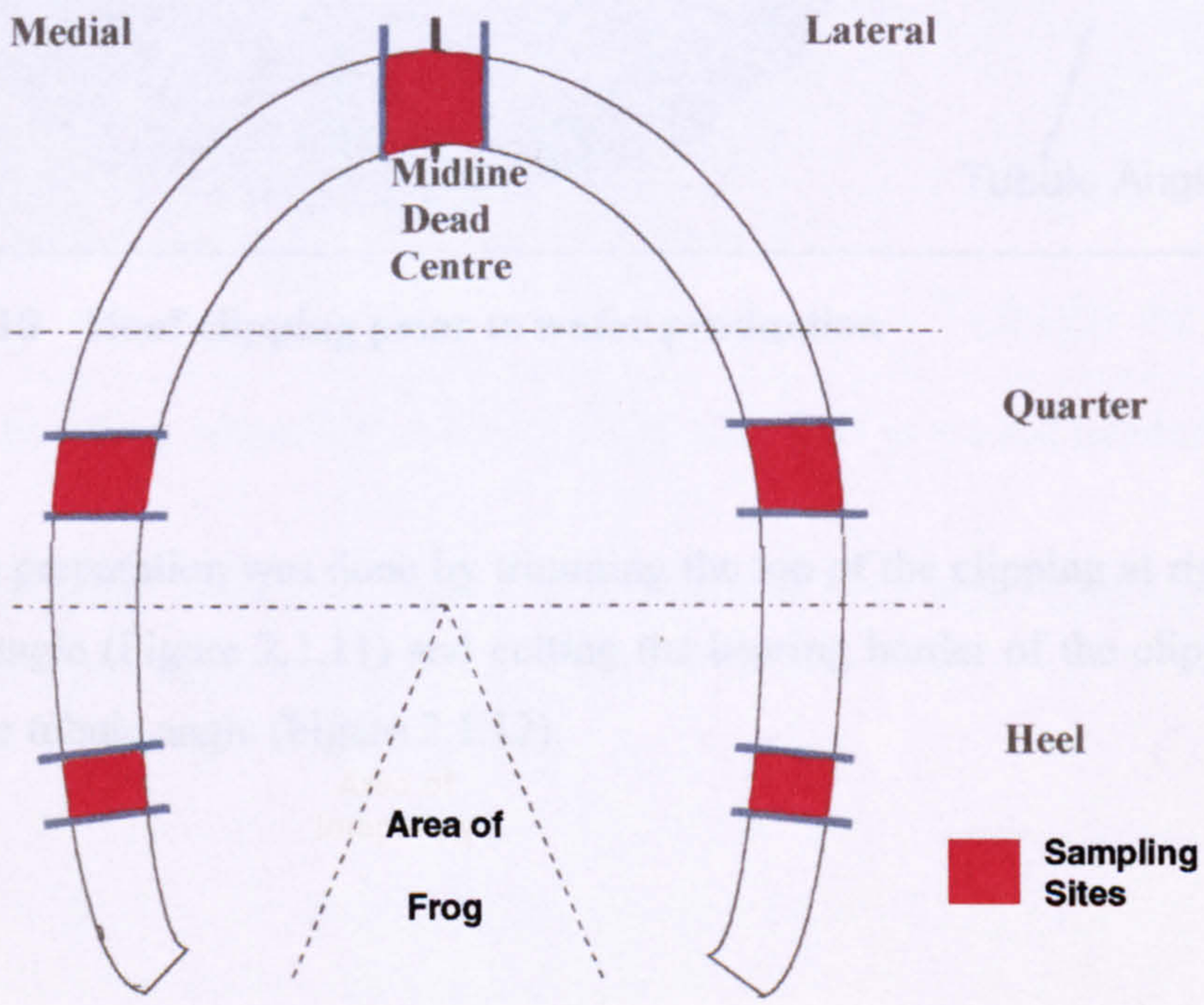


Figure 2.1.9 Hoof clipping sampling sites

Once the clipping section (Figure 2.1.11) was taken from the centre of each sampling area, it was trimmed by eye to provide an approximately 2mm high clipping wafer for analysis (see progression in Figures 2.1.10-2.1.12). As the fresh hoof horn rapidly lost moisture on exposure to the air of the laboratory, it was imperative that all processing was carried out as quickly as possible, to ensure that moisture content analyses reflected the original state as far as was practicable.

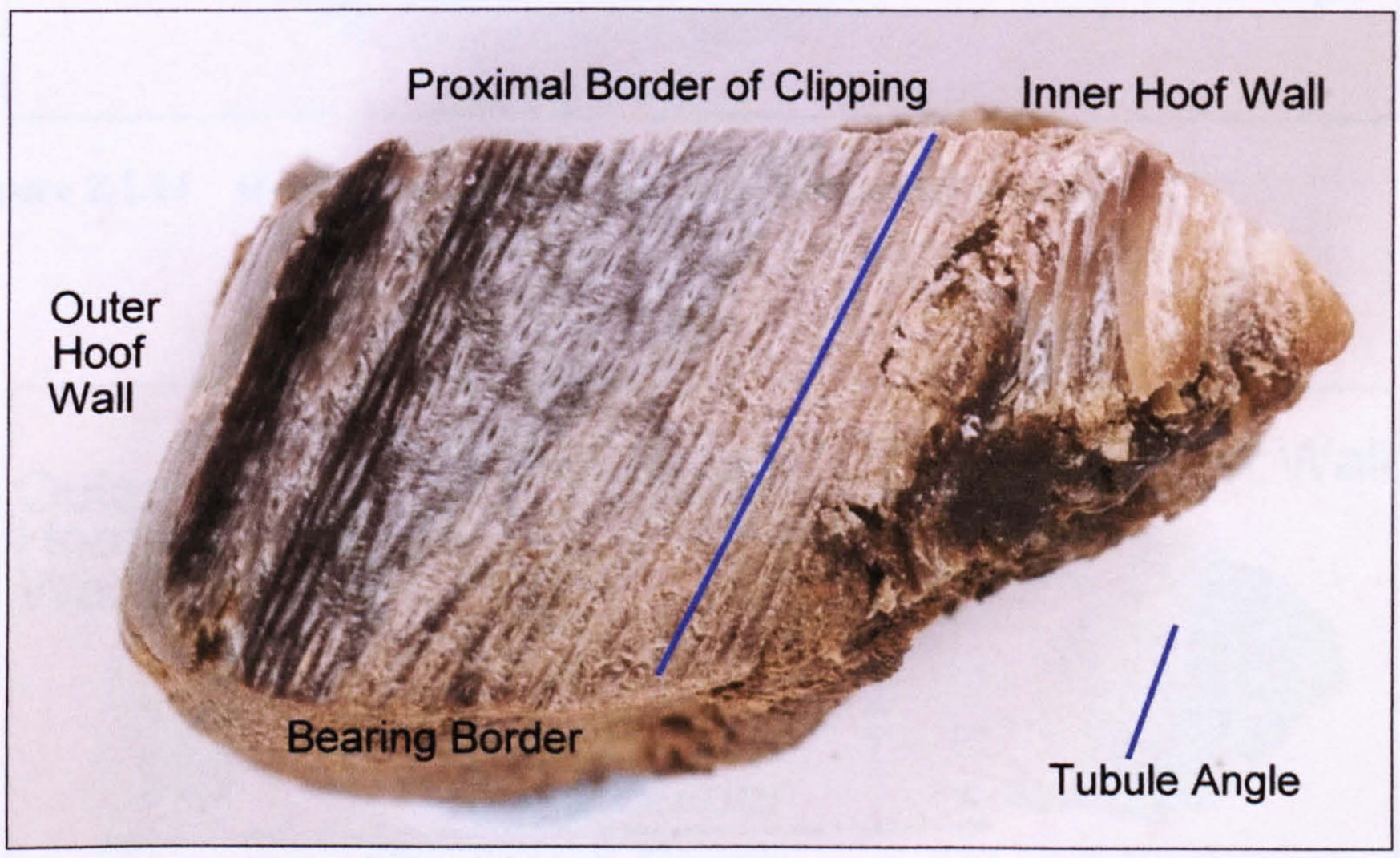


Figure 2.1.10 Hoof clipping prior to wafer production

The sample preparation was done by trimming the top of the clipping at right angles to the tubule angle (Figure 2.1.11) and cutting the bearing border of the clipping at right angles to the tubule angle (Figure 2.1.12).

Figure 2.1.11 Hoof clipping with proximal border trimmed

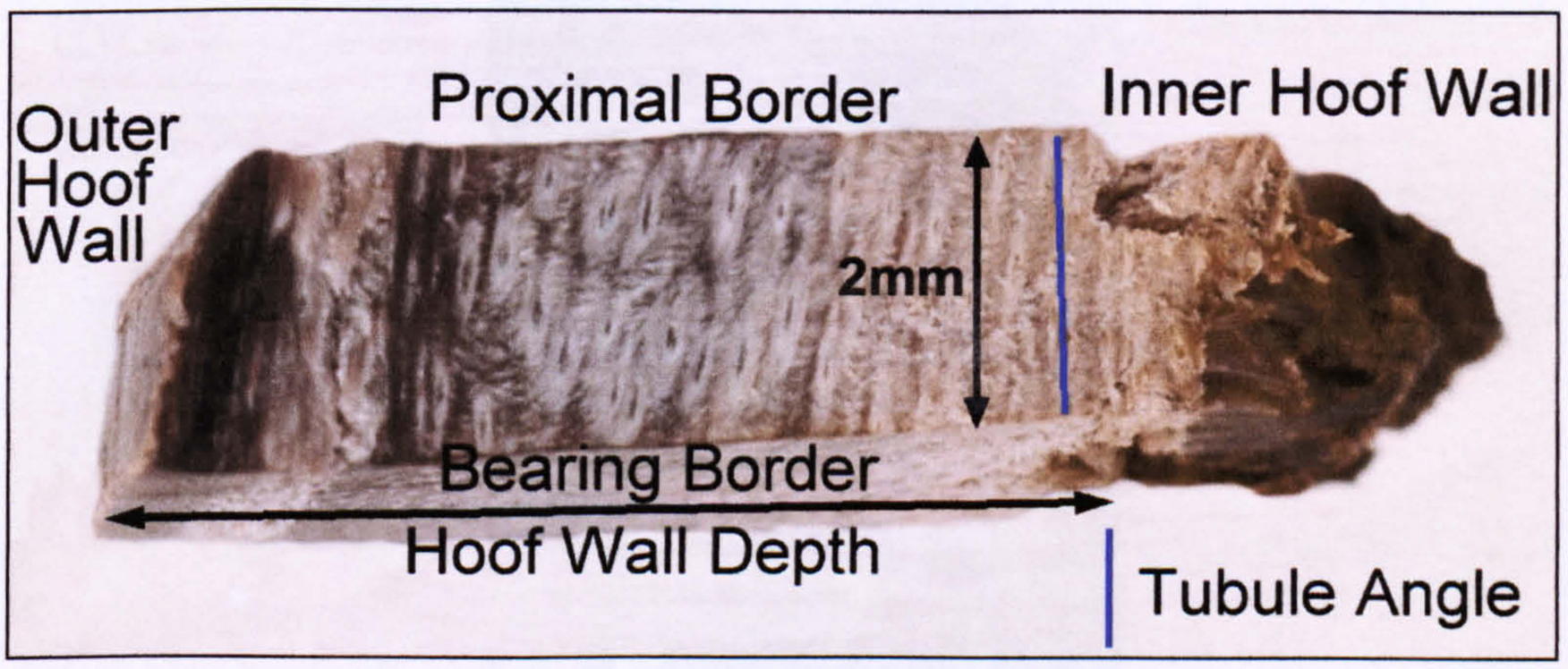


Figure 2.1.12 Hoof clipping with bearing border and proximal border trimmed

This approximately 2mm hoof wall height, full hoof wall depth clipping section was then processed for further analysis.

2.1.4 Hoof wall depth zonal sampling

In the present study, donkey hoof horn was divided into four zones based on visual difference in tubule morphometry (Figure 2.1.14). It was noted that, particularly in donkey hoof horn nearest the *stratum internum,* there tended to be a peculiar arrangement to the tubules which appeared to be in alignment with the laminae of the hoof (zone 4 in Figure 2.1.14). This phenomenon was also described by Bowker (2003), who hypothesised that these particular tubules originate from the laminae, and not from the coronary corium, in direct response to stresses on the hoof structure and termed them laminar derived tubules (LDT) (Figure 2.1.13).

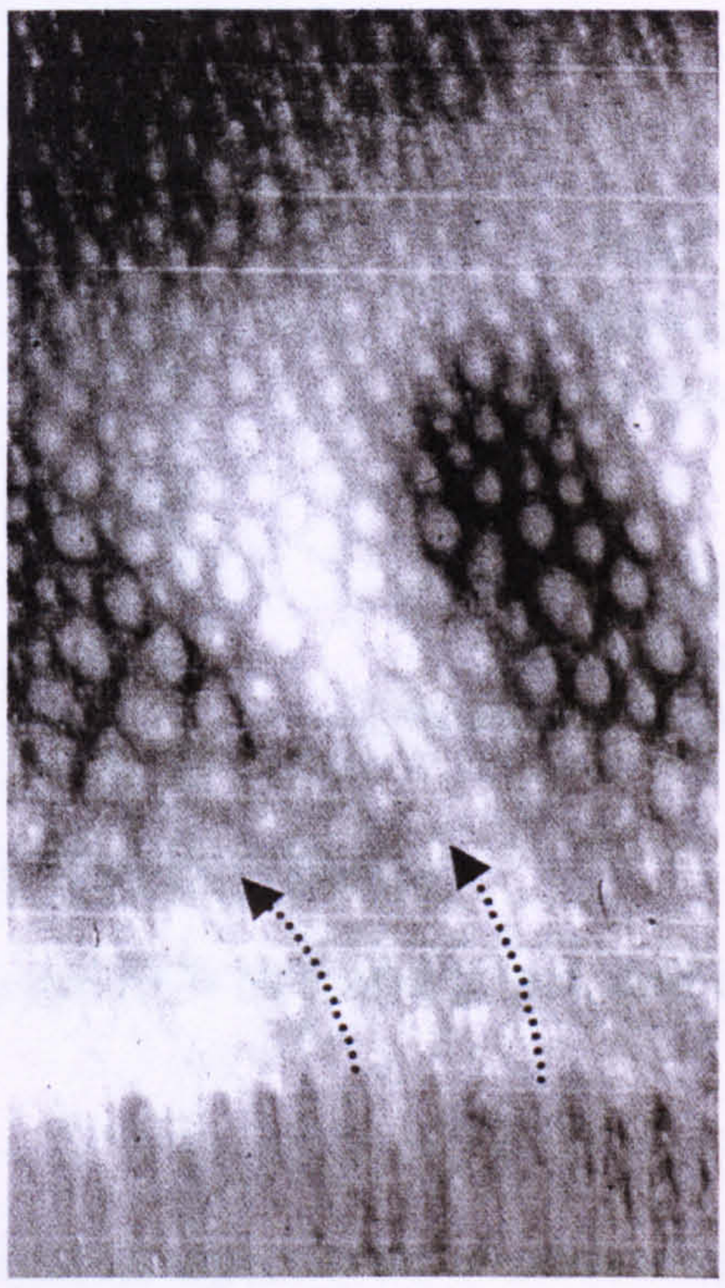


Figure 2.1.13 Inner zone 4 of the *stratum medium* illustrating LDT and the curved path of the tubules (arrows) into the main hoof wall (Bowker, 2003)

It was therefore decided that the *stratum medium* nearest the lamellar junction be designated as zone 4 to investigate any possible effect on moisture content of this change in tubule morphometry and origination.

For zonal hoof wall analysis the hoof wall wafer, taken either from a clipping or morbid hoof at 50% HWH, was then subdivided into four sampling zones (1-4) based on differences in tubule density found by Reilly *et al.*, (1996 & 1998a) and Hopegood (2002). A full hoof wall depth sample (HWD) was also taken. This produced five areas for analysis (Figure 2.1.14).

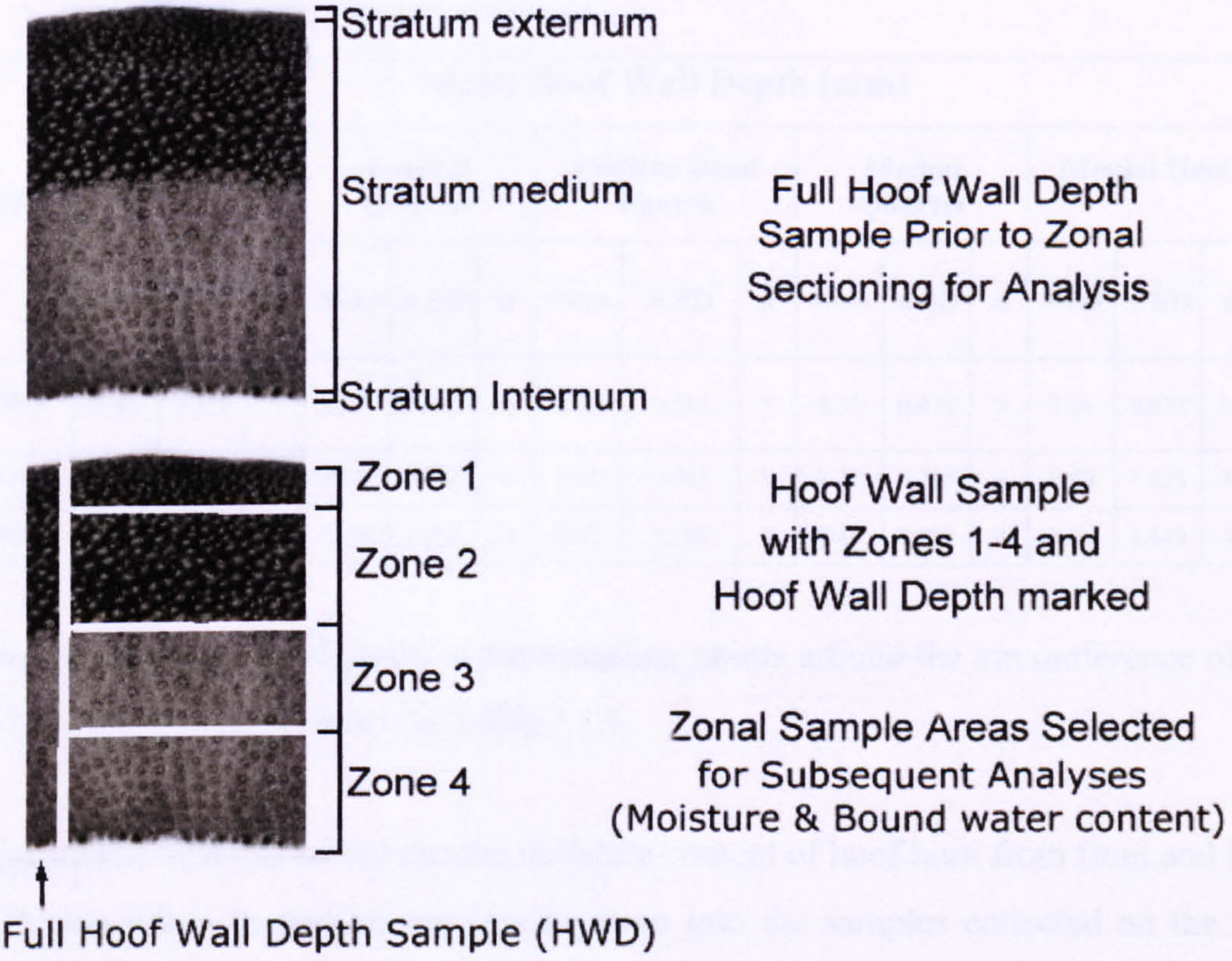


Figure 2.1.14 Hoof wall depth (HWD) wafer intact, and cut into zonal samples

Samples taken were from

- zone 1) the exterior aspect of the *stratum medium*
- zone 2) within the 2nd HWD zone
- zone 3) within the 3rd HWD zone, and
- zone 4) within the 4th HWD of the *stratum medium*.

The samples within zones 2-4 were cut to be the same size as that from zone 1. The depth of the hoof wall varies between individuals due to overall hoof size, and the depth decreases away from the MDC through the quarters towards the heel sections.

Measurements for means of species HWD are shown in Table 2.1.1. The maximum number of individuals sampled for each species was 12, but on occasions, with some of the individuals, there was insufficient hoof growth available to take a clipping sample without risk of injury.

Table 2.1.1 Mean hoof wall depth (mm) at each hoof wall location for each species

Species	Mean Hoof Wall Depth (mm)														
	Lateral Heel			Lateral Quarter			Midline Dead Centre			Medial Quarter			Medial Heel		
	Mean	± SD	n	Mean	± SD	n	Mean	± SD	n	Mean	± SD	n	Mean	± SD	n
Donkey	7.81	0.920	9	8.80	0.625	6	9.50	0.716	5	8.35	0.478	9	7.34	0.823	11
Mule	7.03	1.049	7	8.70	0.882	6	9.01	0.943	6	8.25	0.715	9	6.50	1.025	8
Pony	5.29	1.204	12	7.25	1.661	11	8.02	1.704	9	7.49	1.687	9	5.86	1.449	8

The mean hoof wall depth (mm) at the sampling points around the circumference of the hoof for each species is shown in Table 2.1.1.

No significant difference between the moisture content of hoof horn from front and hind hooves was found in preliminary investigation into the samples collected on the first date in the present study although a difference has been noted in classic studies (Zschokke, 1885). It was therefore decided to analyse the results from the left fore hoof only, as these could then be investigated alongside the results for bound water content and growth from the same hoof.

2.2 Donkey Sanctuary trial animals

The main area of research was an observational study with no specific treatments and no alteration in the animals' day to day husbandry. Within the constraints of such a study, the experimental design was a factorial (2x3x5), three-way mixed design:

Factor 1 = Farm (Brookfield, Town Barton)
Factor 2 = Species (donkey, mule and pony)
Factor 3 = Sampling Time (December, February, May, July, September)

Six donkeys, six mules and six ponies were selected from mixed species groups which were on the same farriery rotation, on each of two Donkey Sanctuary farms. These animals were grouped in weight bands (light, medium and heavy) according to species (Table 2.2.1). Each animal's suitability for trial was based on temperament while being handled and having their hooves trimmed, as quantification of hoof growth required the animals to stand quietly in order for accurate measurements to be taken. Because the study was restricted to using animals in their existing groups, six was the maximum number of ponies and mules which were suitable for the trial at each of the farms.

Mules and ponies are taken into the Donkey Sanctuary only when they are admitted as existing companions to donkeys which are taken into care, hence the low numbers of these animals. Equines are generally acknowledged to have reached adulthood after the age of four. Animals under the age of 4 years and over the age of 35 were excluded from the trial, as young stock have increased hoof growth rates (Butler & Hintz, 1977) and hoof growth rate declines with age (Shannon & Butler, 1979).

Veterinary and movement details were checked to confirm that the animals had been at the Donkey Sanctuary for the previous 6 months to establish baseline conditions, and that they were not suffering from any long term illnesses which could affect the results of the trial. All hooves had been subject to regular trimming and were checked ensure the absence of any hoof deformation or large cracks which may have influenced the hoof growth or moisture content.

All animals were treated as they normally would be by the Sanctuary with regard to feeding, worming and hoof trimming. The study lasted for one year and so the hoof measurement spanned the periods of indoor and outdoor activity of the animals.

During the winter months (November – April) the animals were housed in large indoor barns on deep litter straw bedding, with free access to concrete run out areas, and fed *ad lib* haylage. During the summer months (May-October) the animals were turned out into free-range grass paddocks.

During the trial donkey number 4 (11 years old, 235kg) was lost due to being re-homed. As there were no extra donkeys available at Town Barton, the data from donkey number 15 (12 years old, 211kg) from Brookfield was used as the most suitable substitute to maintain a group of 12 donkeys.

Table 2.2.1 Details of animal population used for Donkey Sanctuary trial

Animal D – Donkey P – pony M – Mule	Farm Location	Sex Gelding (M) Mare (F)	Age at Start of Project (years)	Bodyweight at Start of Project (kg)	Weight Band Light (L) Medium (M) Heavy (H)	Pigmented (P) or Non-Pigmented (NP) Hoof
D1	Town Barton	M	23	152	L	NP
D2	Town Barton	M	15	158	L	P
D3	Town Barton	M	34	178	M	P
D5	Town Barton	F	26	180	M	P
D6	Town Barton	F	12	235	H	P
D7	Brookfield	M	16	150	L	P
D8	Brookfield	M	22	162	L	P
D10	Brookfield	M	23	186	M	P
D11	Brookfield	F	8	189	M	P
D13	Brookfield	F	7	205	H	P
D14	Brookfield	F	15	209	H	P
D15	Brookfield	M	12	211	H	P
P1	Town Barton	M	13	135	L	P
P2	Town Barton	M	17	141	L	P
P3	Town Barton	F	22	142	L	P
P4	Town Barton	F	27	150	M	P
P6	Town Barton	M	14	194	M	NP
P7	Town Barton	F	23	194	M	NP
P8	Brookfield	M	14	136	L	P
P9	Brookfield	M	28	153	M	P
P10	Brookfield	F	19	205	H	P
P11	Brookfield	F	12	231	H	P
P12	Brookfield	F	21	234	H	P
P13	Brookfield	F	20	245	H	P
M1	Town Barton	M	20	230	L	P
M2	Town Barton	M	8	239	L	P
M3	Town Barton	M	16	263	M	P
M4	Town Barton	M	22	264	M	NP
M5	Town Barton	M	26	266	H	P
M6	Town Barton	M	17	267	H	P
M8	Brookfield	M	13	227	L	P
M9	Brookfield	F	24	243	L	P
M10	Brookfield	F	31	248	M	P
M11	Brookfield	M	8	264	M	P
M12	Brookfield	F	17	272	H	P
M13	Brookfield	F	11	335	H	P

2.3 Statistical analysis

All data were analysed using SPSS 14 for Windows (SPSS Inc., Chicago, Illinois 60606). The data sets were checked for normality using normal probability plots. Where appropriate, data were evaluated for reduction using Factor Analysis. General Linear Models were used to analyse multi-factorial data sets. Significance for all analyses was assessed at a minimum of $p < 0.05$.

CHAPTER 3 – MOISTURE CONTENT OF DONKEY, MULE AND PONY HOOF HORN

3.1 Introduction

The primary purpose of the equid hoof is to provide a protective capsule of horn surrounding the sensitive tissues of the foot. When the hoof strikes the ground the specialised components of the hoof capsule, and the structure of the horn itself, work to dissipate the tremendous forces of locomotion (Dyhre-Poulsen *et al.,* 1994).

Hoof function is facilitated primarily by the anatomical structure, although the natural regulation of horn moisture content is of utmost importance, as it has a close relationship with the biomechanical properties of hoof horn (Butler, 1992, Douglas *et al.,* 1996). In cases where hoof horn moisture is reduced below optimal hydration levels (Bertram & Gosline, 1987), it may be possible to aid the natural regulation of hoof horn moisture by application of topical treatments, or by management of the environment (Robertson *et al.*, 1996).

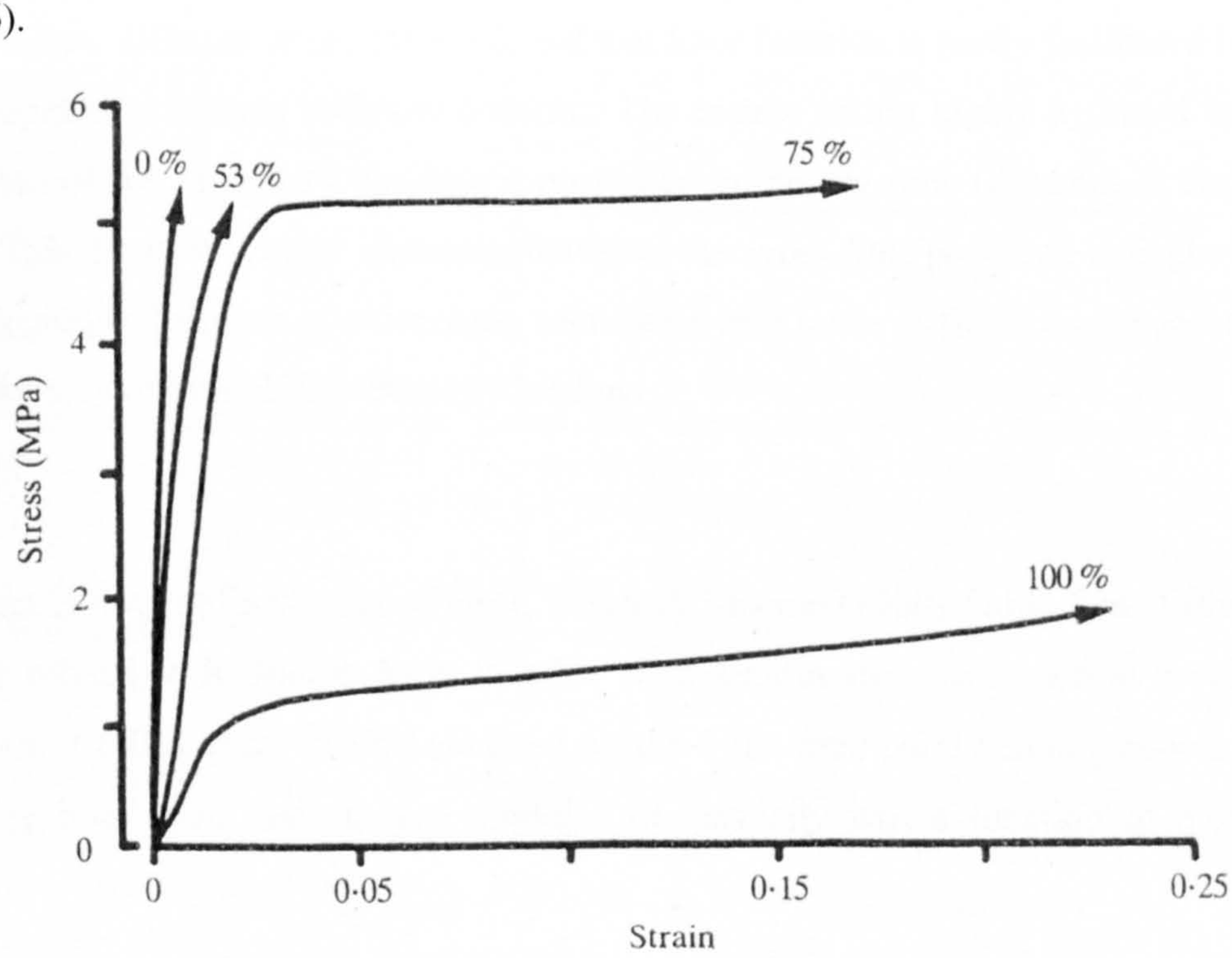


Figure 3.1.1 Tensile tests stressed parallel to the tubule axis at four hydration levels (Bertram & Gosline, 1987).

Bertram & Gosline (1987) found that maximal fracture toughness (22.8kJ/m^2) was demonstrated when excised hoof samples were hydrated within an atmosphere of 75%RH (Figure 3.1.1), which they therefore considered to be the optimal hydration level, as the hoof material was most resilient.

It may be that the donkey, which has evolved to have optimal hoof function in arid conditions (Groves, 1974), may not be very well suited to the wet, temperate climate of the United Kingdom; which could cause over hydration and reduce the effectiveness of the hoof horn. Indeed, Bertram & Gosline (1987) found that fracture toughness was reduced at moisture levels which exceeded 18.2%, this may lead to impaired hoof function.

The precise location and function of water within the hoof horn structure is still under investigation. Butler (1992) stated that tubules are filled with cells that absorb and conduct water. Douglas *et al.*, (1996) found that hoof function is partly facilitated by the natural regulation of horn moisture content. The matrix within highly hydrated keratin lacks much of the secondary bonding it possesses in the dry state (Bertram & Gosline, 1987). This leads to longer distances between the cross-link positions and gives the polymers greater freedom of movement, translating into lower stiffness as the secondary cross links are not available to take any loading.

This effect is not confined to hoof horn, Fraser & Macrae (1980) found that stiffness is inversely related to hydration level in other hard keratin structures such as wool and ovine horn. Collins *et al.*, (1998) obtained results from three point bending experiments on donkey hoof horn that showed modulus of elasticity was a function of moisture content.

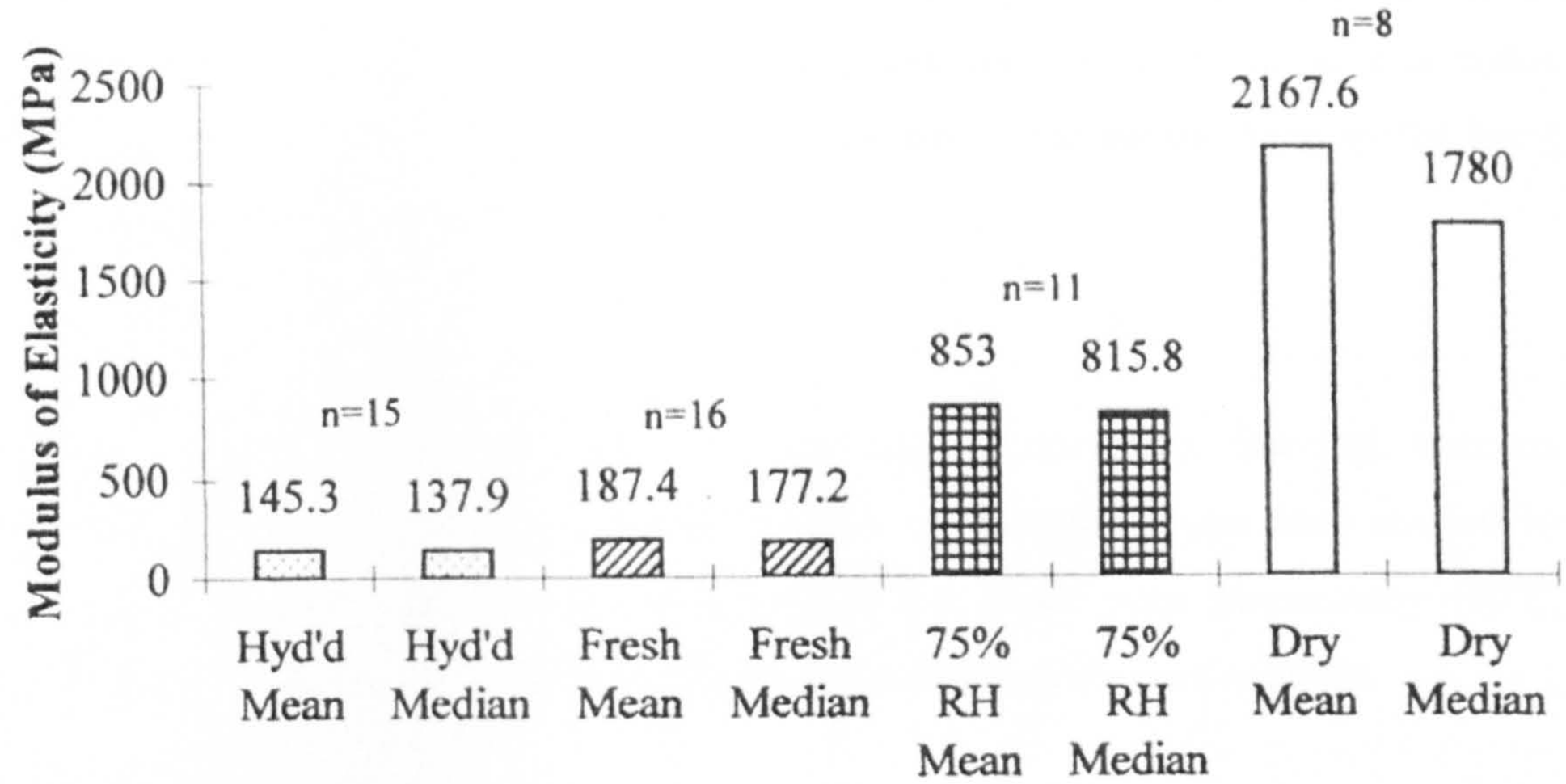


Figure 3.1.2 Comparison of mean and mean modulus of elasticity for sample of donkey hoof horn having different moisture contents (Collins *et al.*, 1998)

The effect of the moisture content on the stiffness of the hoof horn is evident in Figure 3.1.2, where the mean stiffness of the dry samples (2168 MPa) is approximately 11.5 times the mean stiffness of the fresh moisture content samples.

Leach (1980) and Bertram & Gosline (1987) artificially manipulated the moisture content of equine horn and demonstrated a general decrease in stiffness and an increase in maximum extensibility, with increased hydration (Figure 3.1.1).

In contrast, Ley *et al.*, (1998) found that percent moisture (the range for all treatments was 31.16 – 33.83%), as determined by oven drying, was not significantly associated with hoof tensile strength or relative elasticity. This may have been due to their sampling technique, which involved washing twice with deionised water, and samples at 25°C. Both these experimental procedures will have had some effect on their final hoof moisture content results, as hoof horn moisture content is greatly affected by environment (Hayes, 1966) and analysis technique. Ley *et al.*, (1998) also pooled the results from the toe and heel, which will have further affected hoof moisture content

results. Also, the samples were machined prior to testing and the experimental method does not state that the samples were tested in a controlled environment, so it is highly likely that the hoof samples would have lost moisture to the environment whilst being prepared for testing (personal observation).

Techniques used to dry hoof horn, include room temperature, freezing, vacuum, phosphorous pentoxide (P_2O_5), and various oven temperatures have been studied by Hopegood *et al.,* (2002). These authors found that lower oven temperatures (90°C, 100 °C and 105 °C) were inefficient in removing moisture from hoof samples.

Higher drying temperatures are not suitable because the regime can result in conformational changes to the structural proteins (Kuntz & Kauzmann, 1974). Freeze drying and drying over P_2O_5 produced normally distributed data, but freeze drying may affect the mechanical properties of horn. For example, freeze drying has been shown to significantly decrease germination rates in pea and soybean seeds (Vertucci, 1989), which indicates that functional changes in the protein have taken place. Therefore, these findings imply that drying over P_2O_5 is the most suitable technique for hoof horn samples.

3.1.1 Published values for hoof horn moisture content

A wide range of hoof moisture content values has been reported by a number of authors for different sites within the hoof wall, using a variety of drying and sampling techniques (Table 1.3.1 in Section 1.3.1).

Full hoof wall depth *stratum medium* moisture content values for donkey hoof horn was higher at 33% (±SD 2%) from the findings of Hopegood (2002), than that for the horse at 22.7% (±SD 3.4%) (Hinterhofer, 1998). This may contribute to a difference in the mechanics of overall hoof function.

3.1.2 Hoof wall moisture gradients

Various hydration gradients have been reported by different researchers. Leach (1980) and Bertram & Gosline (1987) stated that a vertical gradient existed in horse hoof horn, decreasing from the proximal germinative region to the distal contact surface.

The hoof can be hydrated from within, as systemic water is transferred from the extensive blood and lymph supply of the sensitive structures to adjacent hoof horn cells, and this is then transferred to other hoof horn cells towards the dorsal hoof wall, creating a moisture gradient (Kasapi & Gosline, 1997). Leach (1980) and Douglas *et al.*, (1996) also found a dorso palmar moisture content gradient from the outer hoof wall towards the *stratum internum*. Their work was conducted in horse hoof horn. A similar gradient was found in donkey hoof horn by Hopegood (2002) (Table 1.3.1).

Bertram & Gosline (1987) hypothesised that as the cells moved distally, their hydration state and their properties changed. This is in direct contrast to the findings of Hopegood (2002) who found no significant difference between the moisture content of donkey hoof horn samples taken from 16.7, 33.4, 50.0, 66.7, and 83.4% hoof wall height. This may be indicative of a vertical moisture gradient difference between the two equid species, which would contribute to a difference in overall hoof function. As samples were obtained from clippings in the present study, verification of Hopegood's (2002) findings was not possible.

Douglas *et al.*, (1996) found variation in the modulus of elasticity of equine hoof wall material around the circumference of the hoof. Their moisture content figure of 32.5% for the quarters is greater than observations by other authors for fresh horse hoof moisture content for other regions of the hoof (Table 1.3.1), with the exception of Benedetti (1948) who obtained a moisture content of 36.33% by oven drying. This increased moisture content may be a major contributory factor in higher elasticity levels in the heels.

3.1.3 Rationale

Following preliminary investigations, there was found to be no significant difference ($p > 0.05$) between hoof wall moisture of 50% HWH and hoof clipping samples when fully hydrated. However, there were significant differences ($p < 0.05$) between the moisture content of hoof wall zones 1 and 2, and 3 and 4 in donkey and horse hooves. Fully hydrated hoof horn was used for differential scanning calorimetry samples (Chapter 4), whereas fresh hoof moisture content was studied for the main hoof moisture study in this thesis.

3.1.4 Aim

The aim of this part of the study was to:

- establish the fresh moisture content across the *stratum medium* depth, and full hoof wall depth of donkey and pony hoof horn, and compare the results with those for mule hoof horn.

- establish the fresh moisture content across the *stratum medium* depth of mule hoof horn and thereby ascertain the existence, or not, of a dorso-palmar increase in moisture content for this species

- establish the effect of season on fresh moisture content across the *stratum medium* depth, and full hoof wall depth of donkey, mule and pony hoof horn at five sites around the circumference of the hoof capsule, over a period of 12 months.

3.2 Method

3.2.1 Experimental design

The experimental design was a factorial (2x3x5), three-way mixed design, as described on page 61.

Factor 1 = Farm (Brookfield, Town Barton)

Factor 2 = Species (donkey, mule and pony)

Factor 3 = Sampling Time (December, February, May, July, September)

3.2.2 Fresh moisture content (%)

After removal from storage in Parafilm and trimming as described previously in Chapter 2, the hoof wall was sectioned into four zones and the HWD, as illustrated in Figure 2.1.14. The hoof wall was cut as quickly as possible and was weighed immediately on the Sartorius (Sartorius AG, Germany) balance to minimise any moisture loss. Samples were then placed in a desiccator with phosphorous pentoxide (P_2O_5) for 2 weeks to attain equilibrium at 0%RH (Hopegood, 2002).

Samples were then reweighed and the fresh moisture content (MC %) calculated:

Equation 1

$$MC\% = \frac{{}^{a}\text{Fresh Weight} - {}^{b}\text{Dry Weight}}{{}^{a}\text{Fresh Weight}} \times 100$$

Key for Equation 1

[a] Fresh weight = Fresh mass of sample

[b] Dry weight = Mass of sample following no further mass loss after drying over P_2O_5

Hoof moisture content of fresh hoof clippings were measured over a 12 month period in a controlled trial using donkeys, mules and ponies, kept in mixed groups and maintained in a temperate climate in the UK. Moisture content was determined in clipping samples using five specific locations around the circumference of the hoof wall on the left fore hoof of each animal. These clippings were taken every 10 weeks.

3.2.4 Statistical analyses

All data were analysed using SPSS 14 for Windows (SPSS Inc., Chicago, Illinois 60606). The data sets were checked for normality using normal probability plots. Data was evaluated for reduction using Factor Analysis and then General Linear Models were used to analyse the reduced data set. Significance for all analyses was assessed at a minimum of $p < 0.05$.

3.3 Hoof wall moisture content (MC%) analysis results

Mixed between-within subjects analyses of variance were conducted on the full hoof wall depth samples. There were no significant differences ($p > 0.05$) in hoof wall MC% between months, species, farms, gender or hoof pigment. However, for zonal samples, there were significant differences ($p < 0.05$) in hoof wall MC% between months, species and between zones within the hooves of each species, but not between farms, gender or hoof pigment.

The overall trend in all species was for an increase in the mean MC% from the outer hoof wall in zone 1 (donkey 26.40% [±0.524 SE_D], mule 23.99% [±0.431 SE_D], pony 21.19% [±0.322 SE_D]) to the inner aspect of the *stratum medium* at zone 4 (donkey 38.46% [±0.235 SE_D], mule 36.76% [±0.255 SE_D], pony 32.68% [±1.088 SE_D]. Zones 1 and 2 had lower MC% than zones 3 and 4 for all species on all sampling dates.

Due to the large size of the hoof MC% data sets, factor analysis was performed on the raw data. The full set of hoof wall MC% results are to be found in Appendix 1.

The data are presented in the following order, in an attempt to present them logically and to extract all different comparisons:

- 3.3.1 Factor analysis of raw data
- 3.3.2 General linear model analysis of the effects of species, farm, month, gender and pigment on full hoof wall depth hoof MC%
- 3.3.3 General linear model analysis of full hoof wall depth hoof MC% between months
- 3.3.4 General linear model analysis of the effects of species, farm, month, gender and pigment on zonal hoof MC%
- 3.3.5 General linear model analysis of hoof wall MC% between zones

3.3.1 Factor analysis of the raw data set

Factor analysis was conducted in order to reduce the data set. Analyses were carried out on the whole of the data for each month.

The 20 items of the MC% analysis (4 zonal samples from 5 sample sites of the hoof) for December were subjected to principal components analysis (PCA) using SPSS Version 15. Principal components analysis revealed one component with an eigenvalue exceeding 1, explaining almost 100% of the variance. An inspection of the scree plot revealed a clear break after the first component (Figure 3.3.1).

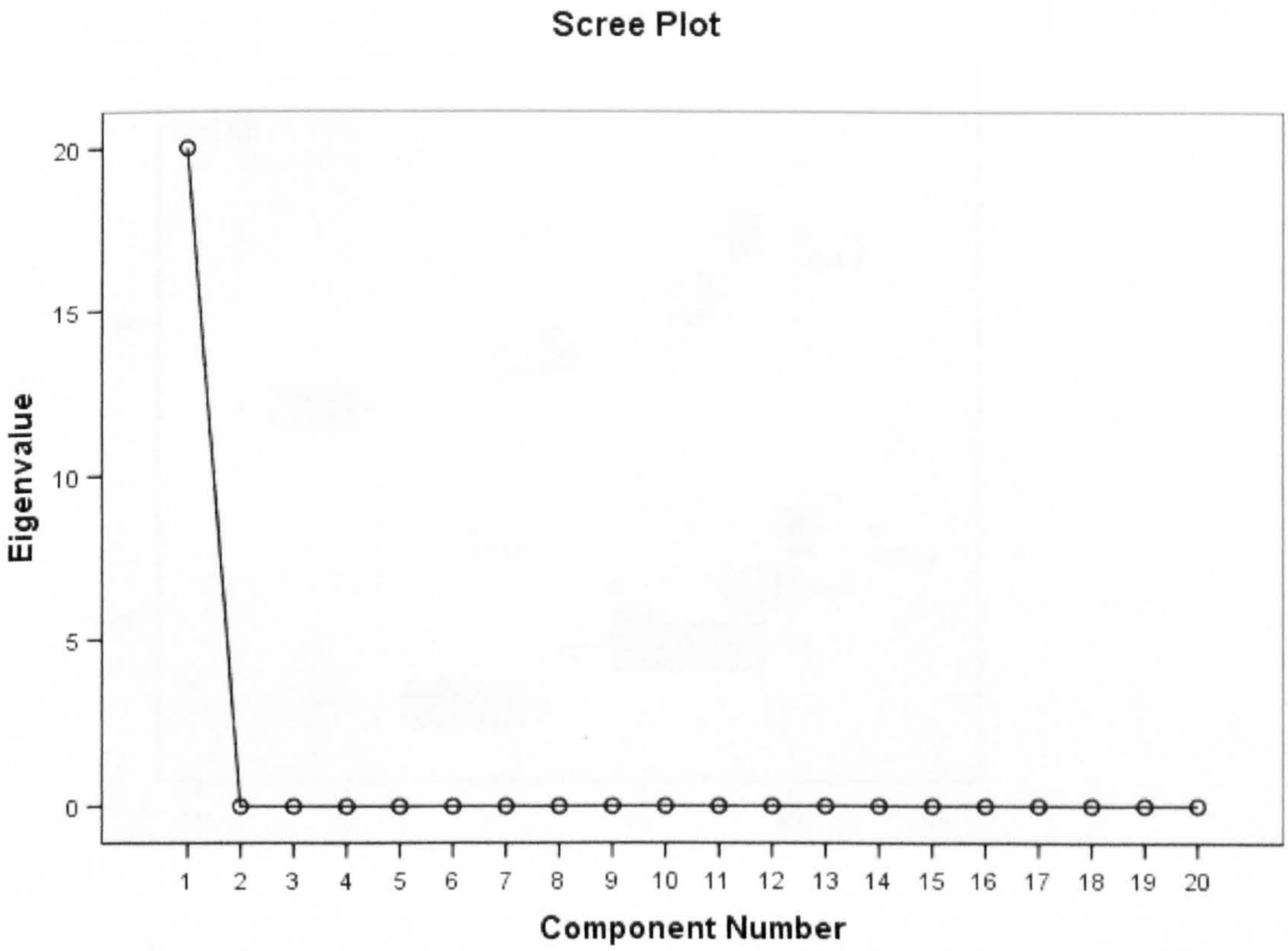


Figure 3.3.1 Scree test plot of hoof wall zonal sample MC% for December

Using Catell's (1966) scree test, it was decided to retain one component for further investigation. This was further supported by the results of Parallel Analysis, which

showed only one component with an eigenvalue exceeding the corresponding criterion values for a randomly generated data matrix of the same size (20 animals x 144 zones).

It was decided that, as one factor loaded on all five sampling points of the hooves, all the MC% results from the sampling points would be meaned.

A similar distribution was obtained for Principal Components Analysis of the MC% results for February, May, July and September. Box plots of the data revealed differences between the MC% of zones of the hoof wall (Figure 3.3.2).

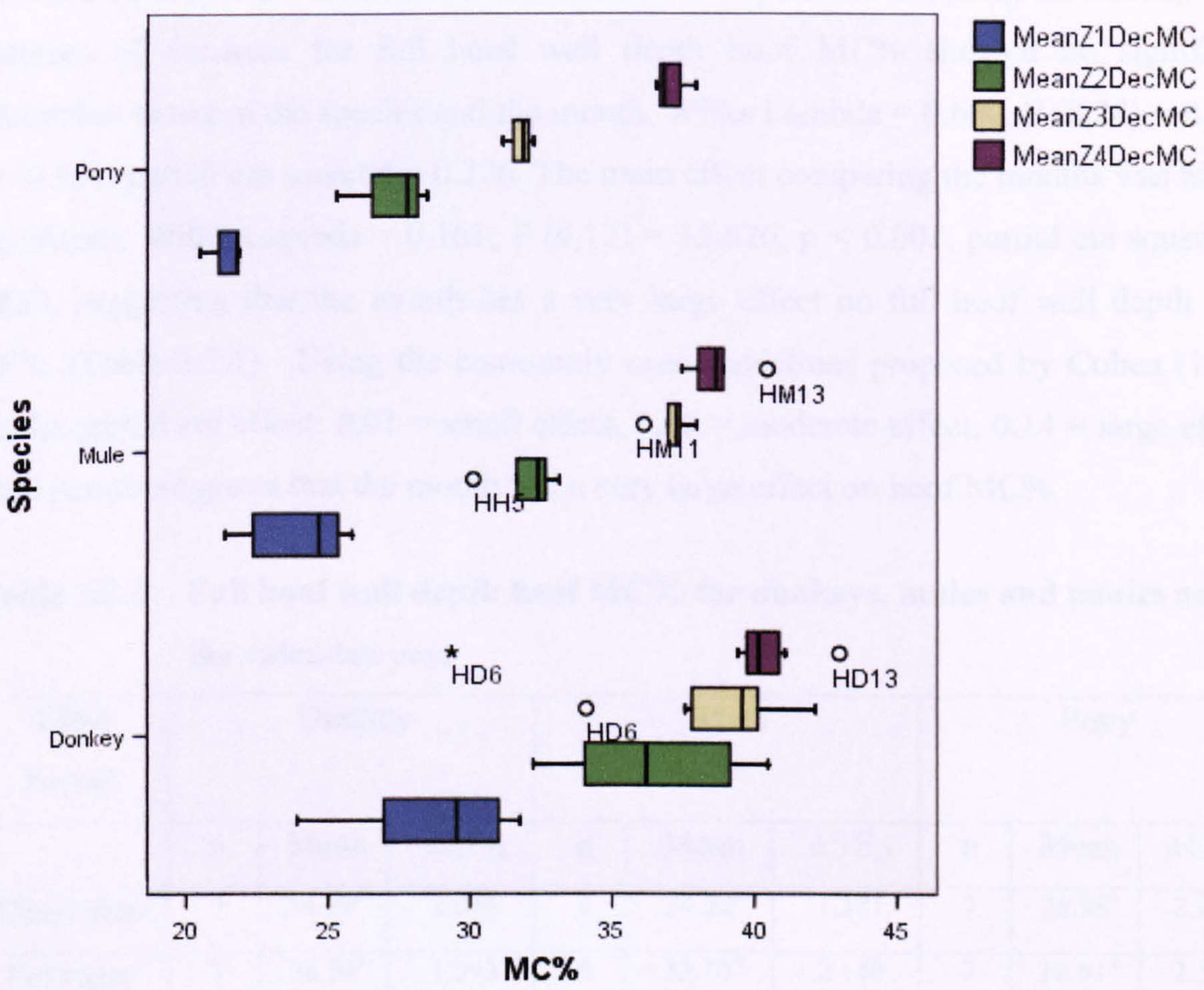


Figure 3.3.2 Boxplot of MC% of hoof wall zonal samples for December for donkeys, mules and ponies

3.3.2 General linear model analysis of the effects of species, farm, month, gender and pigment on full hoof wall depth hoof MC%

Factor analysis of full hoof wall depth samples also showed one factor loaded on all five sampling points of the hooves, so all the MC% results from the sampling points were meaned.

Mixed between-within subjects analyses of variance were conducted on the full hoof wall depth hoof MC% results, to assess the impact of month, farm, sex and species on hoof MC%, across the calendar year (December – September sampling occasions). The analyses of variance for full hoof wall depth hoof MC% showed no significant interaction between the species and the month, Wilks Lambda = 0.600, F (8,24) = 0.551, p = 0.551, partial eta squared = 0.226. The main effect comparing the months was highly significant, Wilks Lambda = 0.161, F (4,12) = 15.626, p < 0.001, partial eta squared = 0.839, suggesting that the month has a very large effect on full hoof wall depth hoof MC% (Table 3.3.1). Using the commonly used guidelines proposed by Cohen (1988) for the partial eta effect: 0.01 = small effect, 0.06 = moderate effect, 0.14 = large effect, these results suggests that the month has a very large effect on hoof MC%.

Table 3.3.1 Full hoof wall depth hoof MC% for donkeys, mules and ponies across the calendar year

Time Period	Donkey			Mule			Pony		
	n	Mean	±SE_D	n	Mean	±SE_D	n	Mean	±SE_D
December	7	36.19^{ac}	2.018	4	34.22^{a}	1.381	7	28.98^{c}	3.706
February	7	36.54^{c}	1.593	4	33.10^{c}	2.140	7	28.61^{c}	2.354
May	7	33.81^{bc}	1.745	4	30.65^{bd}	1.871	7	25.82^{cd}	2.168
July	7	34.24^{c}	2.601	4	32.20^{d}	1.911	7	25.13^{cd}	3.442
September	7	34.58^{ac}	0.986	4	32.44^{ad}	1.924	7	28.14^{cd}	1.947

Full hoof wall moisture content of species, within months, significant difference between means with same superscripts (a = p < 0.05, b = p < 0.005, c, d = p < 0.001) in rows

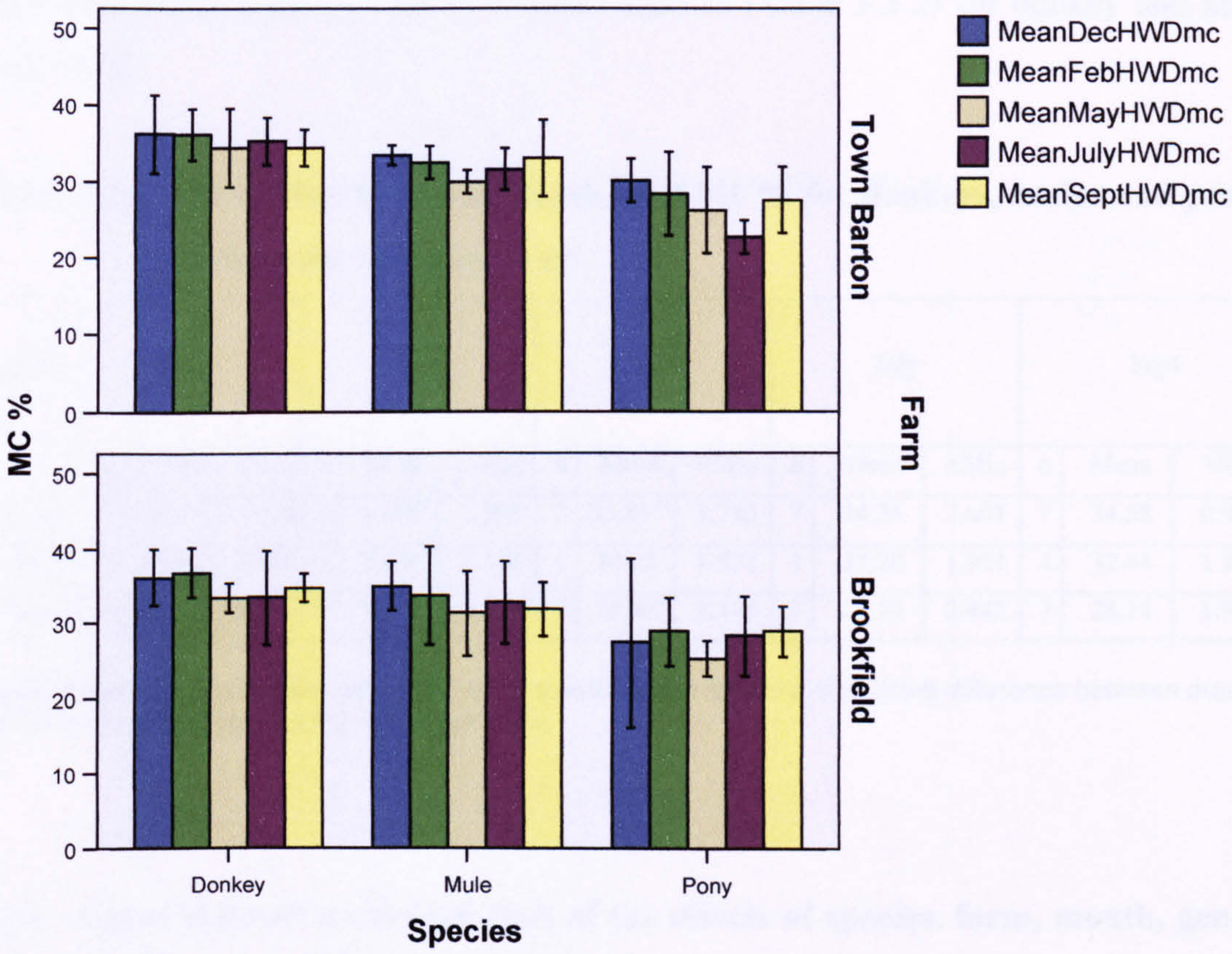


Figure 3.3.3 Comparative full hoof wall depth MC% throughout the calendar year for donkeys, mules and ponies at Brookfield and Town Barton farms

3.3.3 General linear model analysis of full hoof wall depth MC% between months

Mixed between-within subjects analyses of variance was also conducted to assess the impact of month on hoof MC% for the full hoof wall depth samples. There was no significant interaction between month and species, Wilks Lambda = 0.600, F (8,24) = 0.874, p = 0.551, partial eta squared = 0.226. There was, however, a large main effect for month, Wilks Lambda = 0.161, F (4,12) = 15.26, $p < 0.001$, partial eta squared = 0.839.

These results suggest that the month has a very large effect on hoof MC% (Cohen 1988). However, *Post hoc* analysis using the Scheffe test, only revealed a few

significant differences ($p < 0.05$) between months (Table 3.3.2) for donkey and mule hoof MC%.

Table 3.3.2 Mean full hoof wall depth hoof MC% for donkeys, mules and ponies across the calendar year

Species	Dec			Feb			May			July			Sept		
	n	Mean	$\pm SE_D$	n	Mean	$\pm SE_D$	n	Mean	$\pm SE_D$	n	Mean	$\pm SE_D$	n	Mean	$\pm SE_D$
Donkey	7	36.19[a]	2.018	7	36.54[b]	1.593	7	33.81[ab]	1.745	7	34.24	2.601	7	34.58	0.986
Mule	4	34.22	1.381	4	33.10	2.140	4	30.65	1.871	4	32.20	1.911	4	32.44	1.924
Pony	7	28.98[a]	3.706	7	28.61	2.354	7	25.82[a]	2.168	7	25.13	3.442	7	28.14	1.947

Full hoof wall depth moisture content for each month, within species, significant difference between means with same superscripts (a,b = $p < 0.05$) in rows

3.3.4 General linear model analysis of the effects of species, farm, month, gender and pigment on zonal hoof MC%

Mixed between-within subjects analyses of variance were conducted on the zonal hoof MC% results, to assess the impact of farm and species on hoof MC%, for each zone across the calendar year (December – September sampling occasions).

Zone 1

The mixed between-within subjects analyses of variance for zone 1 hoof MC% showed no significant interaction between the species and the month, Wilks Lambda = 0.310, $F(8,20) = 1.988$, $p = 0.102$, partial eta squared = 0.443. All species showed a lowering of zone 1 hoof MC% for the summer months compared with the winter months (Figure 3.3.4).

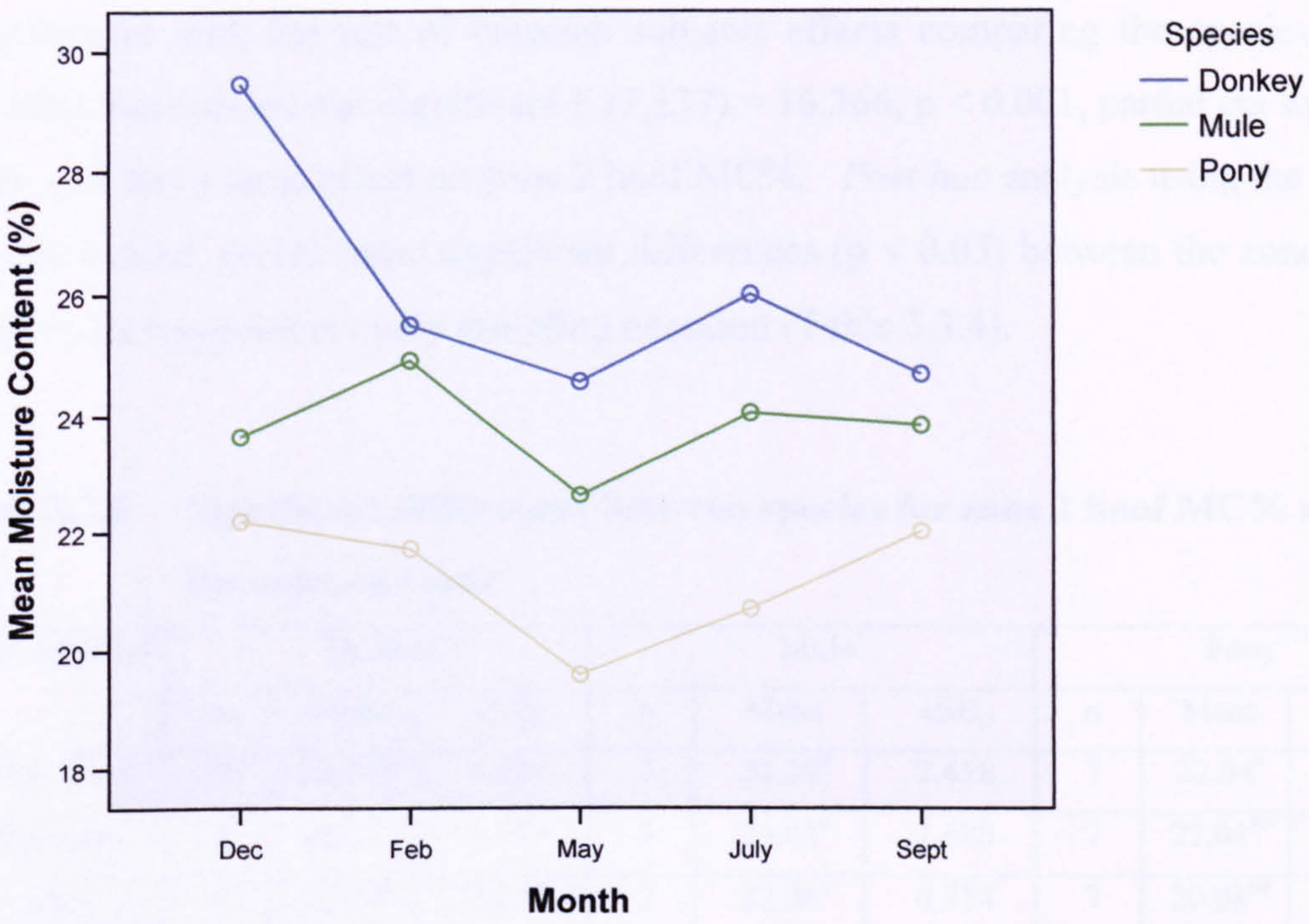


Figure 3.3.4 MC% of zone 1 hoof wall samples throughout the calendar year for donkeys, mules and ponies

Further investigation using *Post hoc* analysis (Scheffe test) of the between months zone 1 hoof MC% for each species revealed that there were significant differences between months for zone 1 MC% for donkeys alone, between December 28.77% (±4.039 SE_D) and May 23.04% (±2.215 SE_D) and September 23.30% (±1.335 SE_D) (Table 3.3.3).

Table 3.3.3 Significant differences between months for zone 1 hoof MC% of donkeys, mules and ponies across the calendar year

Species	Dec			Feb			May			July			Sept		
	n	Mean	±SE_D	n	Mean	±SE_D	n	Mean	±SE_D	n	Mean	±SE_D	n	Mean	±SE_D
Donkey	6	28.77[ab]	4.039	6	25.21	1.777	6	23.04[a]	2.215	6	25.70	3.445	6	23.30[b]	1.335
Mule	3	24.20	2.458	3	23.02	2.680	3	22.26	0.554	3	23.52	0.306	3	23.74	1.330
Pony	7	22.04	1.557	7	22.01	2.247	7	20.08	1.499	7	20.02	2.599	7	21.28	1.078

Zone 1 hoof moisture content for each month, within species, significant difference between means with same superscripts (a,b = $p < 0.05$) in rows

The main effect comparing the species also did not appear to be significant, Wilks Lambda = 0.502, F (4,10) = 2.483, p = 0.111, partial eta squared = 0.498, suggesting that species does not have a significant effect on Zone 1 hoof MC%. This was in disagreement with the test of between subjects effects comparing the species, which indicated that species was significant F (2,137) = 16.366, p < 0.001, partial eta squared = 0.716, and had a large effect on zone 2 hoof MC%. *Post hoc* analysis using the Scheffe test did, indeed, reveal some significant differences (p < 0.05) between the zone 1 hoof MC% of each species at every sampling occasion (Table 3.3.4).

Table 3.3.4 Significant differences between species for zone 1 hoof MC% across the calendar year

Time Period	Donkey			Mule			Pony		
	n	Mean	$\pm SE_D$	n	Mean	$\pm SE_D$	n	Mean	$\pm SE_D$
December	6	28.77^{de}	4.039	3	24.20^{d}	2.458	7	22.04^{e}	1.557
February	6	25.21^{a}	1.777	3	23.02^{b}	2.680	7	22.01^{ab}	2.247
May	6	23.04^{d}	2.215	3	22.26^{a}	0.554	7	20.08^{ad}	1.499
July	6	25.70^{c}	3.445	3	23.52^{a}	0.306	7	20.02^{ac}	2.599
September	6	23.30^{a}	1.335	3	23.74	1.330	7	21.28^{a}	1.078

Zone 1 hoof moisture content of species, within months, significant difference between means with same superscripts (a,b = p < 0.05, c = p < 0.005, d, e = p < 0.001) in rows

Zone 2

The mixed between-within subjects analyses of variance for zone 2 hoof MC% showed no significant interaction between the species and the month, Wilks Lambda = 0.707, F (8,24) = 0.569, p = 0.793, partial eta squared = 0.159. There was, however, a substantial main effect for month. Wilks Lambda = 0.181, F (4,12) = 13.611, p < 0.001, partial eta squared = 0.793. All species certainly showed a reduction in Zone 2 hoof MC% for the summer months compared with those for the winter months (Figure 3.3.5). Test of between subjects effects comparing the species were significant F (2,438) = 33.176, p < 0.001, partial eta squared = 0.816, suggesting that the species has a large effect on zone 2 hoof MC%.

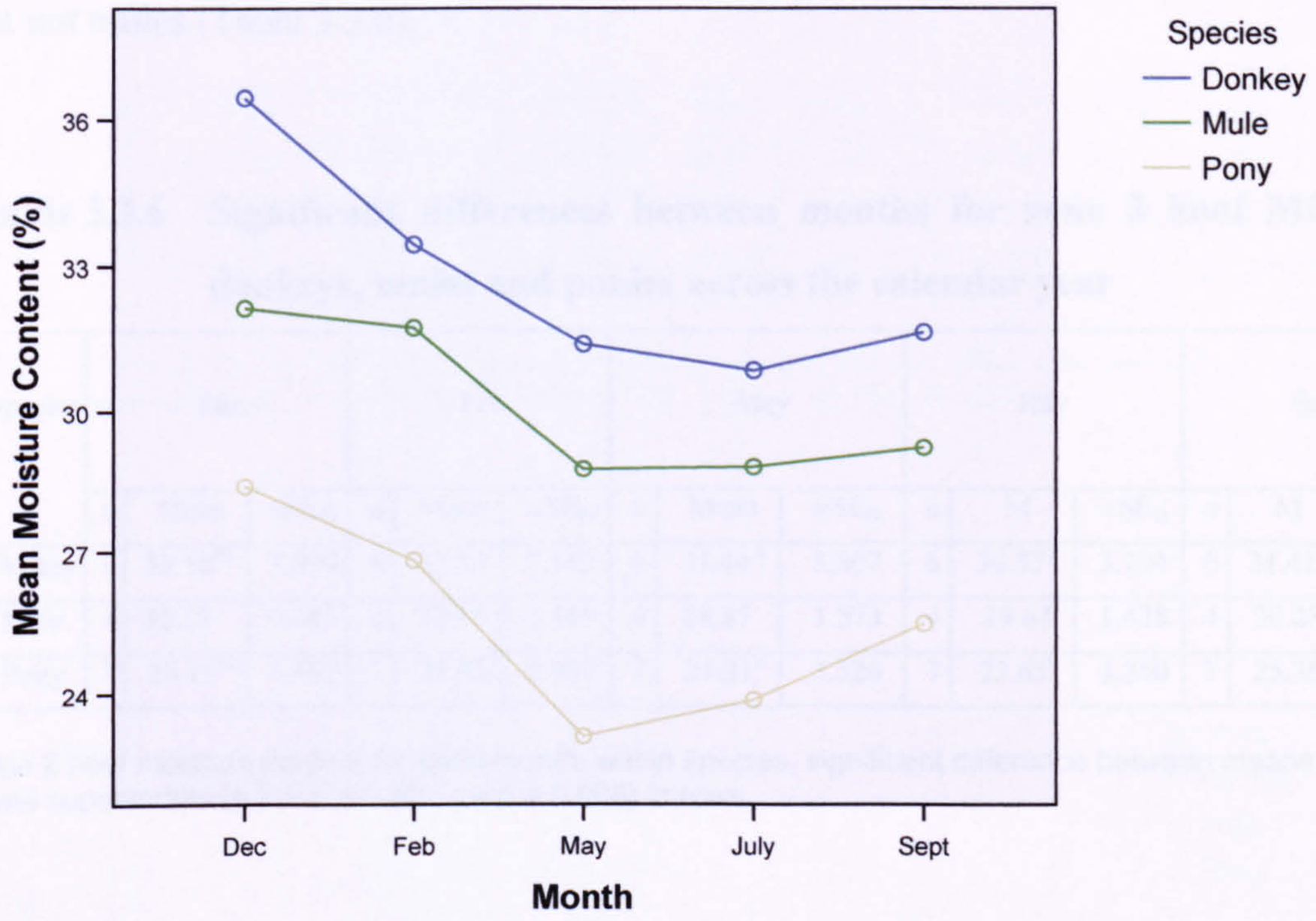


Figure 3.3.5 MC% of zone 2 hoof wall samples throughout the calendar year for donkeys, mules and ponies

Table 3.3.5 details the significant differences ($p < 0.05$) between species hoof MC%, obtained by *post hoc* analysis using the Scheffe test. Whereas, the between subjects test comparing the farms was not significant ($p = 0.869$).

Table 3.3.5 Significant differences between species for zone 2 hoof MC% across the calendar year

Time Period	Donkey			Mule			Pony		
	n	Mean	$\pm SE_D$	n	Mean	$\pm SE_D$	n	Mean	$\pm SE_D$
December	6	36.16^{def}	3.290	4	32.18^{adf}	0.443	7	28.16^{abe}	1.562
February	6	33.13^{d}	2.142	4	32.18^{c}	3.445	7	27.00^{cd}	2.967
May	6	31.44^{ad}	3.307	4	28.87^{ac}	1.573	7	24.21^{cd}	3.3.26
July	6	30.87^{d}	3.104	4	29.64^{c}	1.428	7	23.63^{cd}	3.260
September	6	31.41^{d}	1.102	4	30.28^{a}	1.113	7	25.36^{ad}	1.516

Zone 2 hoof moisture content of species, within months, significant difference between means with same superscripts (a,b = $p < 0.05$, c = $p < 0.005$, d,e,f = $p < 0.001$) in rows

Further investigation using *Post hoc* analysis (Scheffe test) of the between months zone 2 hoof MC% for each species revealed that there were significant differences ($p < 0.05$) in zone 2 hoof MC% between the winter and summer months for donkeys and ponies, but not mules (Table 3.3.6).

Table 3.3.6 Significant differences between months for zone 2 hoof MC% of donkeys, mules and ponies across the calendar year

Species	Dec			Feb			May			July			Sept		
	n	Mean	$\pm SE_D$	n	Mean	$\pm SE_D$	n	Mean	$\pm SE_D$	n	M	$\pm SE_D$	n	M	$\pm SE_D$
Donkey	6	36.16^{abc}	3.290	6	33.13	2.142	6	31.44^{a}	3.307	6	30.87^{c}	3.104	6	31.41^{b}	1.102
Mule	4	32.18	0.443	4	32.18	3.445	4	28.87	1.573	4	29.64	1.428	4	30.28	1.113
Pony	7	28.16^{ac}	1.562	7	27.00	2.967	7	24.21^{c}	3.326	7	23.63^{a}	3.260	7	25.36	1.516

Zone 2 hoof moisture content for each month, within species, significant difference between means with same superscripts (a,b = $p < 0.05$, c = $p < 0.005$) in rows

Zone 3

The mixed between-within subjects analyses of variance for zone 3 hoof MC% showed no significant interaction between the species and the month, Wilks Lambda = 0.360, F (8,24) = 0.569, p = 0.091, partial eta squared = 0.400. There was, however, a substantial main effect for month. Wilks Lambda = 0.121, F (4,12) = 21.721, $p < 0.001$, partial eta squared = 0.879. All species showed a reduction in zone 3 hoof MC% for the summer months compared with those for the winter months (Figure 3.3.6).

Post hoc analysis, using the Scheffe test, of the between months zone 3 hoof MC% for each species revealed that there were significant differences ($p < 0.05$) in zone 3 hoof MC% between the winter and summer months for all three species (Table 3.3.7).

Table 3.3.7 Significant differences between months for zone 3 hoof MC% of donkeys, mules and ponies across the calendar year

Species	Dec			Feb			May			July			Sept		
	n	Mean	$\pm SE_D$	n	Mean	$\pm SE_D$	n	Mean	$\pm SE_D$	n	Mean	$\pm SE_D$	n	Mean	$\pm SE_D$
Donkey	6	38.93^{a}	1.600	6	38.98^{bcd}	1.318	6	36.51^{b}	1.399	6	36.16^{ad}	1.939	6	36.82^{bc}	1.328
Mule	4	37.23^{d}	0.792	4	37.25^{abe}	1.528	4	34.28^{de}	1.767	4	34.67^{a}	1.100	4	35.26^{b}	2.409
Pony	7	33.18^{agf}	1.833	7	32.30^{bd}	3.287	7	27.00^{dg}	2.267	7	26.83^{bf}	4.329	7	29.83^{a}	2.643

Zone 3 hoof moisture content for each month, within species, significant difference between means with same superscripts (a,b,c = $p < 0.05$, d,e = $p < 0.005$, f,g = $p < 0.001$) in rows

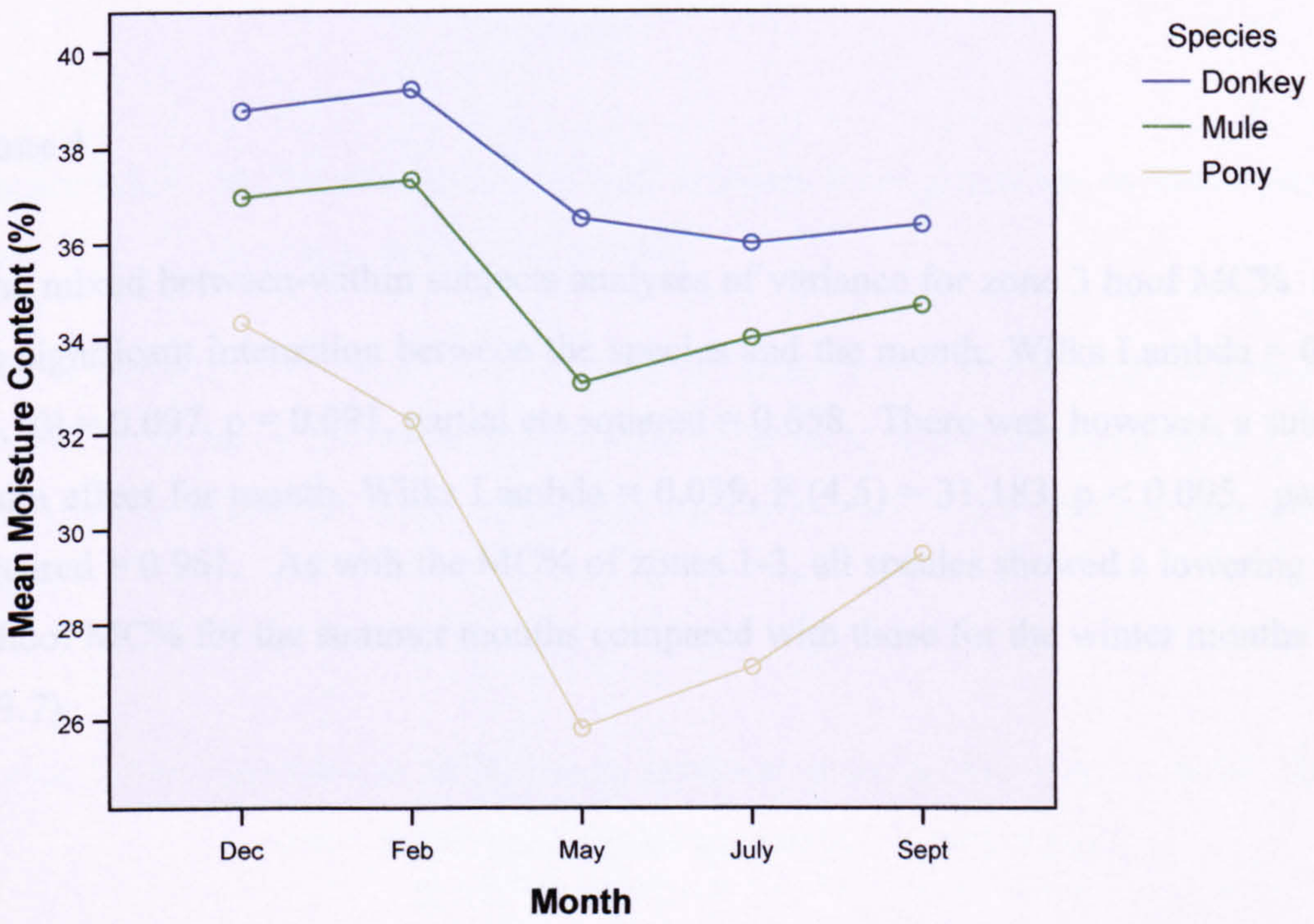


Figure 3.3.6 MC% of zone 3 hoof wall samples throughout the calendar year for donkeys, mules and ponies

Test of between subjects effects comparing the species were significant F (2,546) = 63.478, $p < 0.001$, partial eta squared = 0.894, suggesting that the species has a large

effect on zone 3 hoof MC%, which was borne out by *post hoc* analysis using the Scheffe test (Table 3.3.8).

Table 3.3.8 Significant differences between species for zone 3 hoof MC% across the calendar year

Time Period	Donkey			Mule			Pony		
	n	Mean	$\pm SE_D$	n	Mean	$\pm SE_D$	n	Mean	$\pm SE_D$
December	6	38.93^{d}	1.600	4	37.23^{c}	0.792	7	33.18^{cd}	1.833
February	6	38.98^{d}	1.318	4	37.25^{e}	1.528	7	32.30^{de}	3.287
May	6	36.51^{ad}	1.399	4	34.28^{ae}	1.767	7	27.00^{de}	2.267
July	6	36.16^{d}	1.939	4	34.67^{e}	1.100	7	26.83^{de}	4.329
September	6	36.82^{d}	1.328	4	35.26^{e}	2.409	7	29.83^{de}	2.643

Zone 3 hoof moisture content of species, within months, significant difference between means with same superscripts (a,b = $p < 0.05$, c = $p < 0.005$, d,e = $p < 0.001$) in rows

Zone 4

The mixed between-within subjects analyses of variance for zone 3 hoof MC% showed no significant interaction between the species and the month, Wilks Lambda = 0.117, F (8,10) = 0.097, p = 0.091, partial eta squared = 0.658. There was, however, a substantial main effect for month. Wilks Lambda = 0.039, F (4,5) = 31.183, $p < 0.005$, partial eta squared = 0.961. As with the MC% of zones 1-3, all species showed a lowering of zone 4 hoof MC% for the summer months compared with those for the winter months (Figure 3.3.7).

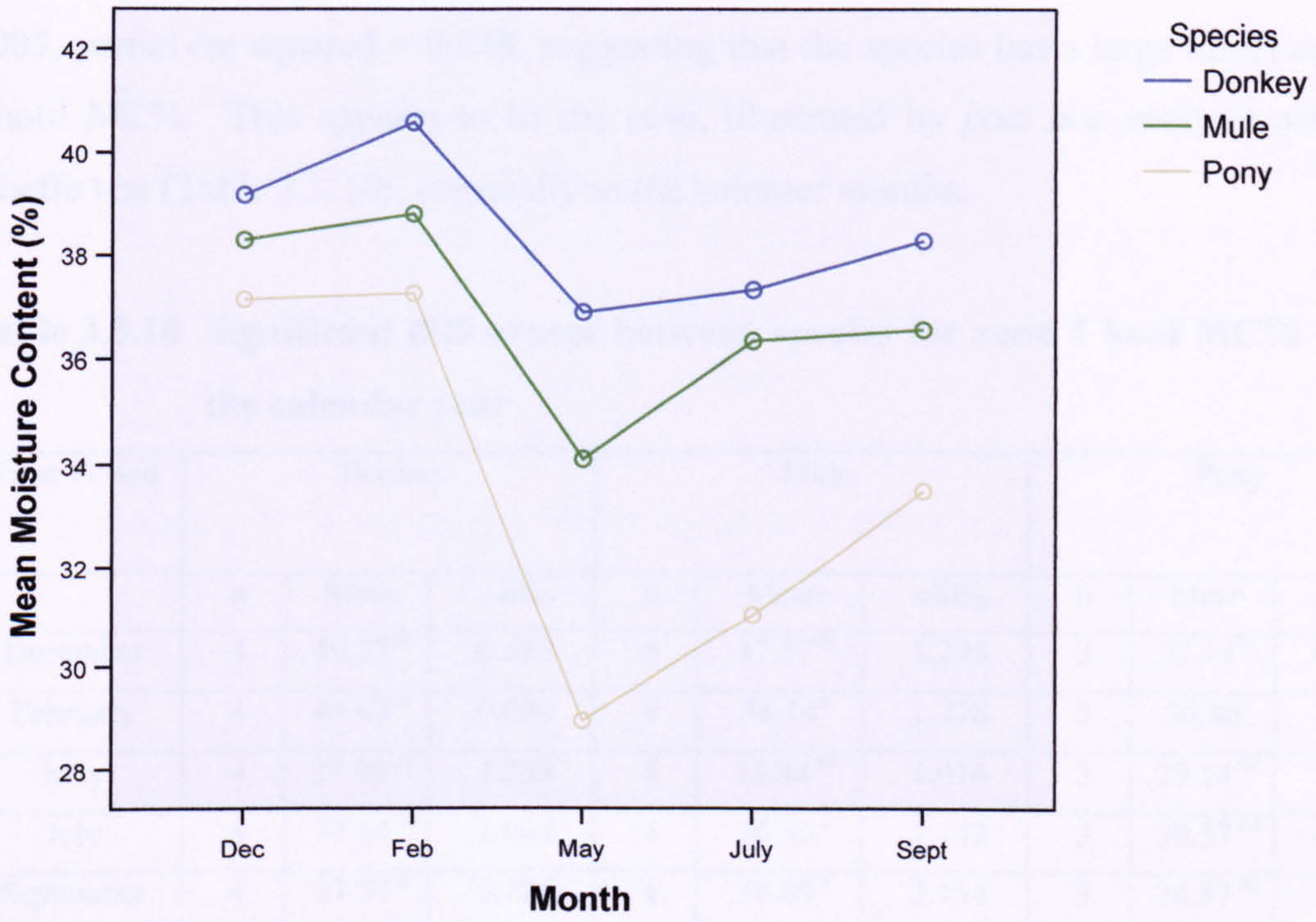


Figure 3.3.7 MC% of zone 4 hoof wall samples throughout the calendar year for donkeys, mules and ponies

Further investigation using *Post hoc* analysis (Scheffe test) of the between months zone 4 hoof MC% for each species revealed that there were significant differences ($p < 0.05$) between the winter and summer months for zone 4 MC% for all three species (Table 3.3.9).

Table 3.3.9 Significant differences between months for zone 4 hoof MC% of donkeys, mules and ponies across the calendar year

Species	Dec			Feb			May			July			Sept		
	n	Mean	$\pm SE_D$	n	Mean	$\pm SE_D$	n	Mean	$\pm SE_D$	n	Mean	$\pm SE_D$	n	Mean	$\pm SE_D$
Donkey	4	40.17	0.683	4	41.03^{ab}	0.620	4	37.08^{a}	1.203	4	37.64^{b}	1.004	4	37.57	0.726
Mule	4	37.57^{c}	1.295	4	38.14^{da}	1.278	4	33.84^{cd}	1.016	4	36.33	1.142	4	36.69^{a}	2.134
Pony	3	37.14^{a}	0.747	3	36.80^{bc}	1.999	3	29.14^{ac}	1.212	3	30.57^{b}	4.207	3	34.87	1.920

Zone 4 hoof moisture content for each month, within species, significant difference between means with same superscripts (a,b = $p < 0.05$, c = $p < 0.005$, d = $p < 0.001$) in rows

The main effect comparing the species was highly significant F (2,107) = 22.324, p < 0.005, partial eta squared = 0.848, suggesting that the species has a large effect on Zone 4 hoof MC%. This appears to be the case, illustrated by *post hoc* analysis using the Scheffe test (Table 3.3.10), especially in the summer months.

Table 3.3.10 Significant differences between species for zone 4 hoof MC% across the calendar year

Time Period	Donkey			Mule			Pony		
	n	Mean	$\pm SE_D$	n	Mean	$\pm SE_D$	n	Mean	$\pm SE_D$
December	4	40.17^{ac}	0.683	4	37.57^{ab}	1.295	3	37.14^{bc}	0.747
February	4	41.03^{ac}	0.620	4	38.14^{a}	1.278	3	36.80^{c}	1.999
May	4	37.08^{ad}	1.203	4	33.84^{ae}	1.016	3	29.14^{de}	1.212
July	4	37.64^{d}	1.004	4	36.33^{c}	1.142	3	30.57^{cd}	4.207
September	4	37.57^{d}	0.726	4	36.69^{a}	2.134	3	34.87^{ad}	1.920

Zone 4 hoof moisture content of species, within months, significant difference between means with same superscripts (a,b = p < 0.05, c = p < 0.005, d,e = p < 0.001) in rows

The General Linear Model analysis results for all zones showed that gender and pigment on had no significant effect (p > 0.05) zonal hoof MC%.

3.3.5 General linear model analysis of hoof wall MC% between zones

The mixed between-within subjects analyses of variance was conducted to assess the impact of species on hoof MC%, across four hoof zones (1-4). There were significant interactions between zones and species, Wilks Lambda = 0.388, F (6,56) = 5.657, $p < 0.001$, partial eta squared = 0.377. The significant interaction means that the impact of the zone on hoof moisture content is influenced by the species, and so general conclusions in the line of main effects are not appropriate, although there was indeed a substantial main effect for zones, Wilks Lambda = 0.016, F (3,28) = 557, $p < 0.001$, partial eta squared = 0.984. Using the commonly used guidelines proposed by Cohen (1988) for the partial eta: 0.01 = small effect, 0.06 = moderate effect, 0.14 = large effect, these results suggests that the zone has a very large effect on hoof MC% (Figure 3.3.7). *Post hoc* analysis using the Scheffe test revealed a large number of significant differences ($p < 0.05$) between zones (Table 3.3.11), with the least significant differences between zones 3 and 4 for all three species.

Table 3.3.11 Mean zonal hoof MC% for donkeys, mules and ponies

Species	Zone 1			Zone 2			Zone 3			Zone 4		
	n	Mean	$\pm SE_D$	n	Mean	$\pm SE_D$	n	Mean	$\pm SE_D$	n	Mean	$\pm SE_D$
Donkey	12	26.40^{def}	0.524	12	32.99^{def}	0.581	12	37.52^{de}	0.350	12	38.46^{de}	0.235
Mule	11	23.99^{def}	0.431	11	30.20^{def}	0.575	11	35.25^{de}	0.342	11	36.76^{de}	0.255
Pony	12	21.19^{bd}	0.322	12	25.14^{bce}	0.521	12	29.24^{acd}	0.657	10	32.68^{ade}	1.088

Hoof moisture content of zones, within rows, significant difference between means with same superscripts (a = $p < 0.05$, b,c = $p < 0.005$, d,e,f = $p < 0.001$) in rows

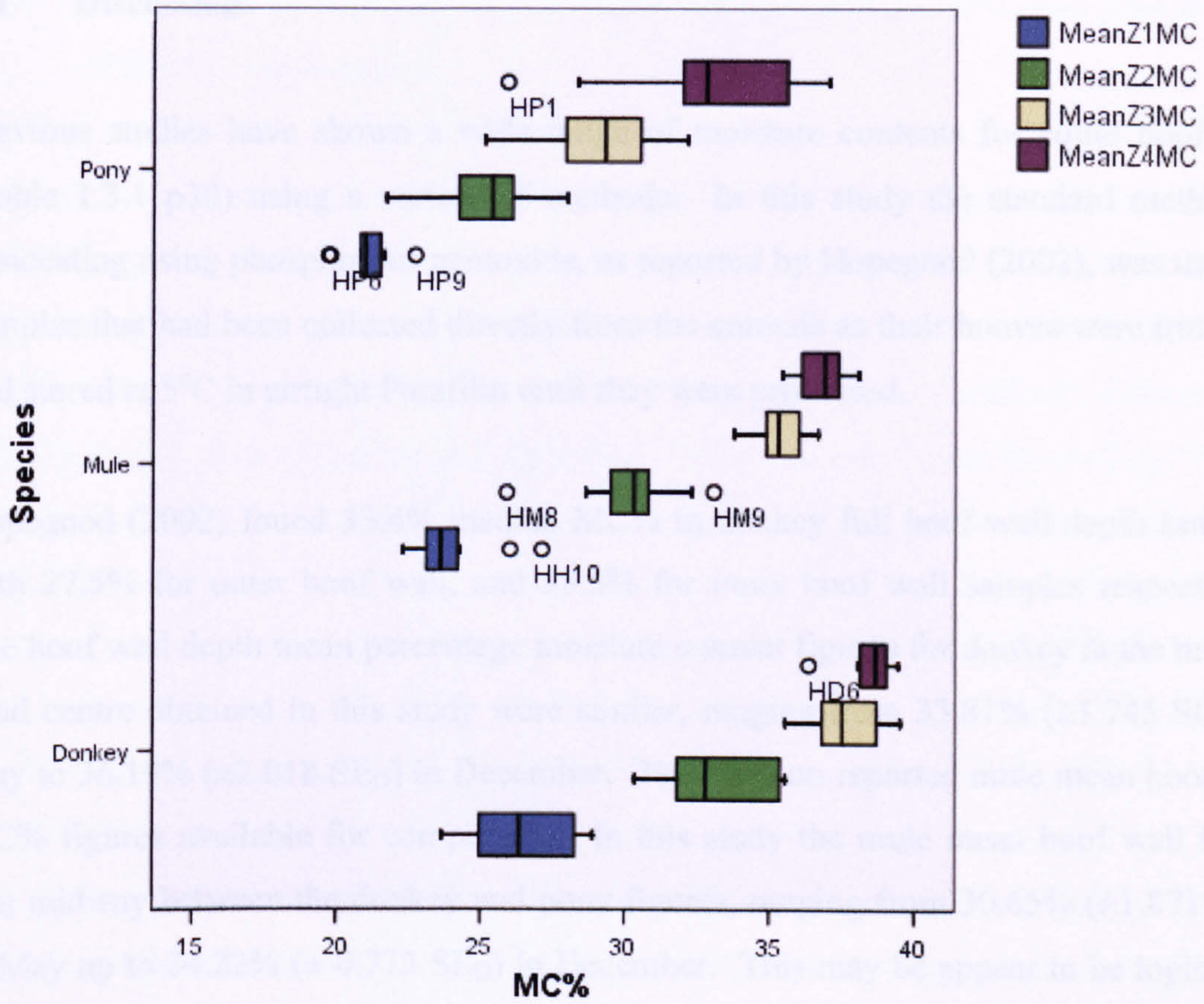


Figure 3.3.8 Boxplot of mean zonal hoof MC% for donkeys, mules and ponies

3.4 Discussion

Previous studies have shown a wide range of moisture contents for equid hoof horn (Table 1.3.1 p30) using a variety of methods. In this study the standard method of desiccating using phosphorous pentoxide, as reported by Hopegood (2002), was used on samples that had been collected directly from the animals as their hooves were trimmed, and stored at 5°C in airtight Parafilm until they were processed.

Hopegood (2002) found 33.4% median MC% in donkey full hoof wall depth samples, with 27.5% for outer hoof wall, and 38.5% for inner hoof wall samples respectively. The hoof wall depth mean percentage moisture content figures for donkey at the midline dead centre obtained in this study were similar, ranging from 33.81% (±1.745 SE_D) in May to 36.19% (±2.018 SE_D) in December. There are no reported mule mean hoof wall MC% figures available for comparison. In this study the mule mean hoof wall MC% was midway between the donkey and pony figures, ranging from 30.65% (±1.871 SE_D) in May up to 34.22% (± 0.773 SE_D) in December. This may be appear to be logical, as the majority of the mules at the Donkey Sanctuary are a hybrid of a donkey jack and a pony mare, but Matthews *et al.*, (1997) reported physiological differences between species and hybrids in which the mule results were not predictably mid-way between the values for donkey and horse. Burnham (2002) identified that mules vary widely in genetic makeup and that the build and type of the mare (dam) of the mule usually dictates the type of work the mule is bred for. Although it is scientific fact that both parents will contribute genetic material to the mule offspring (Burnham 2002), the assumption that the values obtained for mule hoof parameters in this study should lie some way between those for donkey and pony is unconfirmed in the literature. Pony full hoof wall depth percentage hoof wall MC% at the midline dead centre ranged from 25.13% (±3.442 SE_D) in July up to 28.98% (± 3.706 SE_D) in December. These figures are not dissimilar to those of Miyaki *et al.*, (1974), Naumann (1984) and Kainer (1989) for fresh MC% of horse hoof horn full hoof wall depth samples (Table 1.3.1 p31). The similarity between the findings of this study and those of previous authors, who used morbid samples, suggests that hoof clippings are a feasible non invasive method of establishing hoof MC% *in vivo* in equid species.

3.4.1 Differences in hoof wall depth MC% between farms, sexes and hooves with different pigmentation

General linear model analysis showed no significant effect on hoof wall depth MC% from farm location (Figure 3.3.3), sex of animal or pigmentation of the hoof. The indication that the pigmentation of the hoof had no significant effect on the MC% of the hoof horn is in agreement with Benedetti (1948) who found no significant difference in MC% between dark (36.21%) and light (36.47%) pigmented horse hooves; and Leach (1980) who also found no significant difference ($p > 0.05$) in MC% between black and white horse hooves.

3.4.2 Differences in hoof wall depth MC% between months

The level of MC% within the hoof horn is affected by external as well as internal factors. A lower relative humidity (%) in the external environment could cause the hoof to lose water to the environment, as is seen in the results for the summer months (Tables 3.3.2 to 3.3.5, and Figures 3.3.3-3.3.6). This lowering of hoof MC% with changing seasons is seen throughout the hoof wall zones, from the outer zone 1 to the inner zone 4, for all species. This loss of water would have consequence for the locomotion of the animal, as keratin is plasticised by water, and so hoof MC% affects the mechanical properties of the hoof wall (Hopegood 2002).

It is possible that the summer months, with drier conditions (generally lower RH%), provide a better environment for the hoof horn to function, than the wetter winter months. It is hypothesised that lameness and disease are more common when hooves have a higher MC% as Borderas *et al.,* (2004) discovered for cattle. This is in concurrence with the *in vivo* findings of Bertram & Gosline (1987) who considered the optimal hydration and maximal fracture toughness of excised equine hoof samples to occur at 75%RH. One area for further study would be to record the incidence of lameness due to hoof disease and to analyse the MC% of the hooves of the lame animals. This could then be compared to MC% when the animal is sound.

Donkey

The overall trend for donkey HWD mean MC% was to decrease from December to May (Table 3.3.2) and then to increase again to September. December (36.19% ±2.018 SE_D) and February (36.54% ±1.593 SE_D) had significantly higher ($p < 0.05$) mean MC% than May (33.81% ±1.745 SE_D). There were no other significant differences between donkey HWD mean MC% for the subsequent months. Mean figures for summer hoof MC% fluctuated between 33.81% ±1.745 SE_D in May and 34.24% ±2.601 SE_D in July. Decreased MC% could have been due to decreased RH% of the environment, as it is well documented that hooves lose water to the environment at a lower RH% (Bertram and Gosline, 1987). This is investigated in this thesis in greater detail in Chapter 6. The hoof can also be hydrated from within, and it may be that some alteration in the physiological parameters of the donkey, such as hydration status, could result in reduced moisture transfer from the blood and lymph supply of the sensitive structures to adjacent hoof horn cells.

Mule

The mule HWD mean MC% also decreased from December to May (Table 3.3.2) and then increased again to September. Although the December mule hoof samples had the highest mean MC% (34.22% ±1.381 SE_D), there was no significant difference ($p > 0.05$) between the results for December and the other months, of which May showed the lowest hoof MC% at 30.65% ±1.871 SE_D.

Pony

The pony HWD mean MC% also decreased from December to May, but this decrease continued in July, down to 25.13% ±3.442 SE_D before increasing again to September (28.14% ±1.947 SE_D) (Table 3.3.2). Once again, December pony hoof samples had the highest mean MC% (28.98% ±3.706 SE_D), and this was significantly higher ($p < 0.05$)

than the result for May (25.82% ±2.168 SE_D). Pony full hoof wall depth MC% showed greater variability within months, than either donkey or mule full hoof wall depth MC%. The variability within the July pony hoof MC% figures reported (minimum 21.94%, maximum 28.31%) probably affected the significance of the difference between the results for July and December in pony hoof MC%.

Few data are available regarding controlled trials which study hoof MC% variations throughout the seasons. Moisture was one parameter studied by Ley *et al.*, (1998) but no data were presented concerning specific variations over the trial period of one year. The only reference made to the MC% results was that they obtained a relatively narrow range of results with co-efficients of variation between 4-12%. They did scrub all hooves with deionised water prior to hoof clipping sample collection, so this is likely to have had an adverse effect on the validity of their hoof MC% results. However, the co-efficients of variation are similar in the present study between 5-11% for full hoof wall depth samples.

3.4.3 Differences in hoof wall depth MC% between species

The mean hoof wall depth MC% results are discussed in this section. As described in section 3.3.2, factor analysis of full hoof wall depth samples showed one factor loaded on all five sampling points of the hooves, so all the MC% results from the sampling points were meaned.

For full hoof wall depth MC% samples, donkey hoof had significantly higher ($p < 0.05$) MC% than both mule and pony hoof for all months (Table 3.3.1) except July, when donkey hoof only had significantly higher ($p < 0.05$) MC% than pony hoof. The finding of donkey hoof having significantly higher MC% than pony hoof was in agreement with Hopegood (2002), who found donkey horn MC% to be 33%, and significantly higher than that of horse hoof horn at 26% ($p < 0.01$).

Mules had significantly higher mean hoof wall depth MC% than ponies ($p < 0.05$) from February to September inclusive (Table 3.3.1). The difference may also have been significant between mule and pony hoof in December, had there been less variation in the pony hoof MC% (28.98% ± 3.706 SE_D).

As samples were taken from animals that were housed in the same facilities, under the same conditions of care and nutrition, the significant differences shown may be due to intrinsic differences in species hoof wall microanatomy, or hoof wall thickness.

3.4.4 Differences in zonal hoof MC% between farms, sexes and hooves with different pigmentation

As with the full hoof wall depth MC%, a general linear model analysis showed no significant effect ($p > 0.05$) on hoof wall depth MC% from farm location (Figure 3.4.1), sex of animal or pigmentation of the hoof, so the samples were pooled for further analysis of the effect of month and species on zonal hoof MC%.

3.4.5 Differences in zonal hoof MC% between months

Zone 1

When the MC% results from the individual zone 1 analysis are investigated (Table 3.3.3), they reveal that the December donkey MC% (28.77% ± 4.039 SE_D) is significantly higher ($p < 0.05$) than that in the summer months of May (23.04% ± 2.215 SE_D) and September (23.30% ± 1.335 SE_D). Although the summer months also show lower MC% for zone 1 hoof horn for mules and ponies, the difference is not significant ($p > 0.05$). This seasonal variation in hoof MC% would correspond to increased precipitation in autumn and winter, and consequently wetter environmental conditions

for the animals. Although the animals were housed in the winter months, they were allowed free access to external concrete yards, and therefore the external surface of the hoof horn will have been subjected to the prevailing weather conditions at the time of sampling.

Zone 2

The seasonal effect on donkey hoof wall mean MC% is not restricted to the outer zone of the hoof wall, as significant differences ($p < 0.05$) in monthly MC% are also seen in zone 2 (Table 3.3.6). Here, in zone 2, the significant differences ($p < 0.05$) between hoof MC% in calendar months for not only donkeys, but also for ponies. It is curious, therefore, that similar differences are not seen in mule hooves in zone 2 between months, as being a hybrid of the other two species, it would be expected that the hoof horn would have similar hydration properties. Since the numbers were quite low (donkey $n = 6$, mule $n = 4$ and pony $n= 7$) in the present trial, due to missing samples for some months, it may be worth revisiting this section of work.

Zone 3

There is widespread significant variation ($p < 0.05$) in hoof MC% between the months for all species in zone 3 (Table 3.3.7), even though the inner hoof wall is said to be hydrated from the inner tissues within the dermis of the hoof (Collins *et al.*, 1998). So it may be that the microarchitecture of the hoof wall influences the capacity for change in hoof moisture content, due to zone 3 having a larger proportion of intertubular horn matrix than zones 1 and 2 for all species. The uptake of water in keratins by the matrix is thought to be considerably higher than the uptake by the intermediate filaments (Fraser *et al.*, 1972). For this theory to be fully tested a further study would be required in order to analyse the tubular/intertubular composition of the hoof, through histological studies, while also monitoring the water content of each morphological fraction.

Zone 4

The seasonal effect on hoof wall MC% continues in zone 4 of the hoof wall, as significant differences ($p < 0.05$) in monthly MC% are also seen between months for all species (Table 3.3.9). While this may be caused by external influences, there may also be some biological or nutritional interactions which affect the MC% of the hoof. For example Frackowiak & Komosa (2006) noted that temperature and day length caused changes in the metabolic function and hormonal balance of Konik horses when they studied hoof growth in that species. Mallinson *et al.,* (2006) found a significant interaction ($p < 0.05$) between the lipid and moisture content of sheep claw horn while researching a nutritional supplement. In the present study, it may be that dietary fluctuations caused by the animals being inside on conserved forage in winter, changing to fresh grass in the spring and summer, has significant effects on the composition of the hoof wall in all zones. MC% fluctuations could also be due to stresses on the hoof structure giving rise to laminar derived tubules, which are a feature of the zone 4 hoof morphology (Bowker, 2003), and may affect the moisture holding capacity of zone 4. Further investigation is required to examine any possible interaction between moisture content and laminar derived tubules, by combining histological studies with monitoring of the moisture content of each morphological fraction.

3.4.6 Differences in zonal MC% between species

Zone 1

All species have zone 1 hoof wall MC% between 20% and 29% throughout the year (Table 3.3.4). As all three species were maintained within the same environment, is might be predicted that they would have similar hoof wall zone 1 MC%. Donkey zone 1 hoof had a significantly higher MC% than pony hoof ($p < 0.05$) for all months, and mule

zone 1 hoof also had significantly higher MC% than pony hoof zone 1 for the months of February, May and July. This is contrary to Hopegood (2002) who did not find any significant difference ($p > 0.05$) between donkey and horse hoof MC% in zone 1. There were no significant differences ($p > 0.05$) between donkey and mule zone 1 hoof MC% throughout the year. This may indicate that the zone 1 hoof microanatomy and biochemistry of the two species are very similar. The gross anatomy of the hoof structure within zone 1 in both donkeys and ponies (Figures 1.2.16 and 1.2.17) is characterised by small oval tubules, a small amount of intertubular horn and high tubule density. This similarity in gross appearance indicates that the factors contributing to the significant differences in moisture holding capacity of zone 1 are likely to be at molecular level.

Zone 2

Further into the hoof wall, in zone 2, there were similar significant difference in hoof MC%, with donkey and mule zone 2 hoof having significantly higher ($p < 0.05$) MC% than pony zone 2 hoof for all months (Table 3.3.5). The difference between species zone 2 hoof MC% is emphasised in Figure 3.3.5, which shows pony MC% to be consistently between 4 and 8% lower than donkey and mule throughout the year.

Zone 3

The species differences are further illustrated by the pattern of significant differences in zone 3 (Table 3.3.7), where again donkey and mule zone 3 hoof had significantly ($p < 0.05$) higher MC% than pony zone 3 hoof for all months. Data in Figure 3.3.6 highlights the similarity between the effect of month on zone 3 hoof MC% of donkeys and mules, while the pony zone 3 hoof MC% was consistently 5-9% lower than the other two species.

Zone 4

Zone 4 hoof MC% results show similar significant differences between species (Table 3.3.10) to those seen in zones 2 and 3, although as can be seen in Figure 3.3.6, there was more significant variation ($p < 0.05$) between zone 4 hoof MC% for mule and donkey, and the variation between pony and the other two species was less consistent. The significant difference ($p < 0.05$) between species zone 4 MC% could indicate species variations with regard to internal hydration of the hoof. Many differences exist in physiological parameters between the donkey and the horse (Burnham, 2002), and these may well contribute to the blood and lymph supply of the sensitive structures to adjacent hoof horn cells, which could subsequently affect the internal hydration of the *stratum medium*. This is an area of anatomical comparison which would be worth investigating further, particularly with regard to digital blood pressure. There may also be between species differences in zone 4 laminar derived tubules, which may affect the MC% of zone 4. An additional histological investigation, to examine any potential between species differences in the distribution of laminar derived tubules, would be required to substantiate any hypothesis of this nature.

The finding that donkey hoof horn in each of the zones had significantly ($p < 0.05$) higher MC% than pony hoof horn was in agreement with Hopegood (2002) who found that donkey hoof horn MC% for zones 2 to 4 was significantly higher ($p < 0.01$) than that of horse hoof horn. It could be argued that the differences Hopegood (2002) found were due to nutritional intake or to environmental difference because in that case, the donkeys were located at the Donkey Sanctuary in Devon, and the horses at the Friends of Bristol Horse Society near Bristol, 70 miles away. This is not the case in the present study, as for this part of the analyses, all species were housed together in groups, fed the same diet. No results have previously been reported for mule hoof horn MC%.

3.4.7 Differences between zonal moisture content within species

As a general trend, for all species, the hoof horn MC% increases in an anterior-posterior direction towards the internal hoof structures, away from the outer hoof wall (Table 3.3.11). This gradient is in agreement with previous studies (Douglas *et al.*, 1996; Hopegood, 2002) and the moisture gradient is fundamental to the hoof wall function and mechanical stability (Kasapi & Gosline, 1999). There were significant differences ($p < 0.05$) between the outer zones 1 and 2, and the inner zones 3 and 4, for all species. There were significant ($p < 0.001$) rises in MC% from zone 1 (26.40% ±0.524 SE_D) to zone 4 (38.46 ±0.235 SE_D) in donkey hoof, for mule hoof (zone 1 = 23.99% ±0.431 SE_D to zone 4 = 36.76 ±0.255 SE_D) and pony hoof (zone 1 = 21.19% ±0.322 SE_D to zone 4 = 32.68 ±1.088 SE_D).

The pony hoof wall MC% showed a gradual increase in MC% from zone 1 to zone 4 with interzonal increases of 3.95% (±1.87 SE_D) between zones 1 and 2, 4.10% (±2.07 SE_D) between zones 2 and 3, and 3.44% (±2.96 SE_D) between zones 3 and 4. Whereas the mule hoof MC% showed distinct divisions between zones 1 and 2 (6.21% ±2.19 SE_D) and 2 and 3 (5.05% ±2.03 SE_D) and then an increase of only 1.51% (±1.71 SE_D) between zones 3 and 4. The interzonal increases in MC% across the zones of donkey hoof were similar to those of mule, with large increases in MC% between zones 1 and 2 (6.59% ±1.74 SE_D) and 2 and 3 (4.53% ±2.77 SE_D) and then a smaller increase of 0.94% (±1.54 SE_D) between zones 3 and 4. This comparison may further strengthen the argument that mule hoof horn is closer in composition to the hoof horn of the donkey than the pony. Further investigation of the tubule density, morphology and biochemistry of mule hoof horn is needed to establish whether this hypothesis is valid.

The significance of the mean difference between zones 1 and 2 ($p < 0.005$) of pony hoof MC% is in contradiction to the research of Hopegood (2002), who found no significant difference ($p > 0.05$) in hoof horn MC% between zones 1 and 2 of horse hoof. She did, however, find significant differences ($p < 0.05$) in hoof MC% between zones 2, 3 and 4 for donkey hoof horn, which is in agreement with the results in the current trial where

differences were highly significant ($p < 0.001$) (Table 3.3.11). The literature review provided no previous figures, either zonal or full hoof wall depth, for mule hoof MC%.

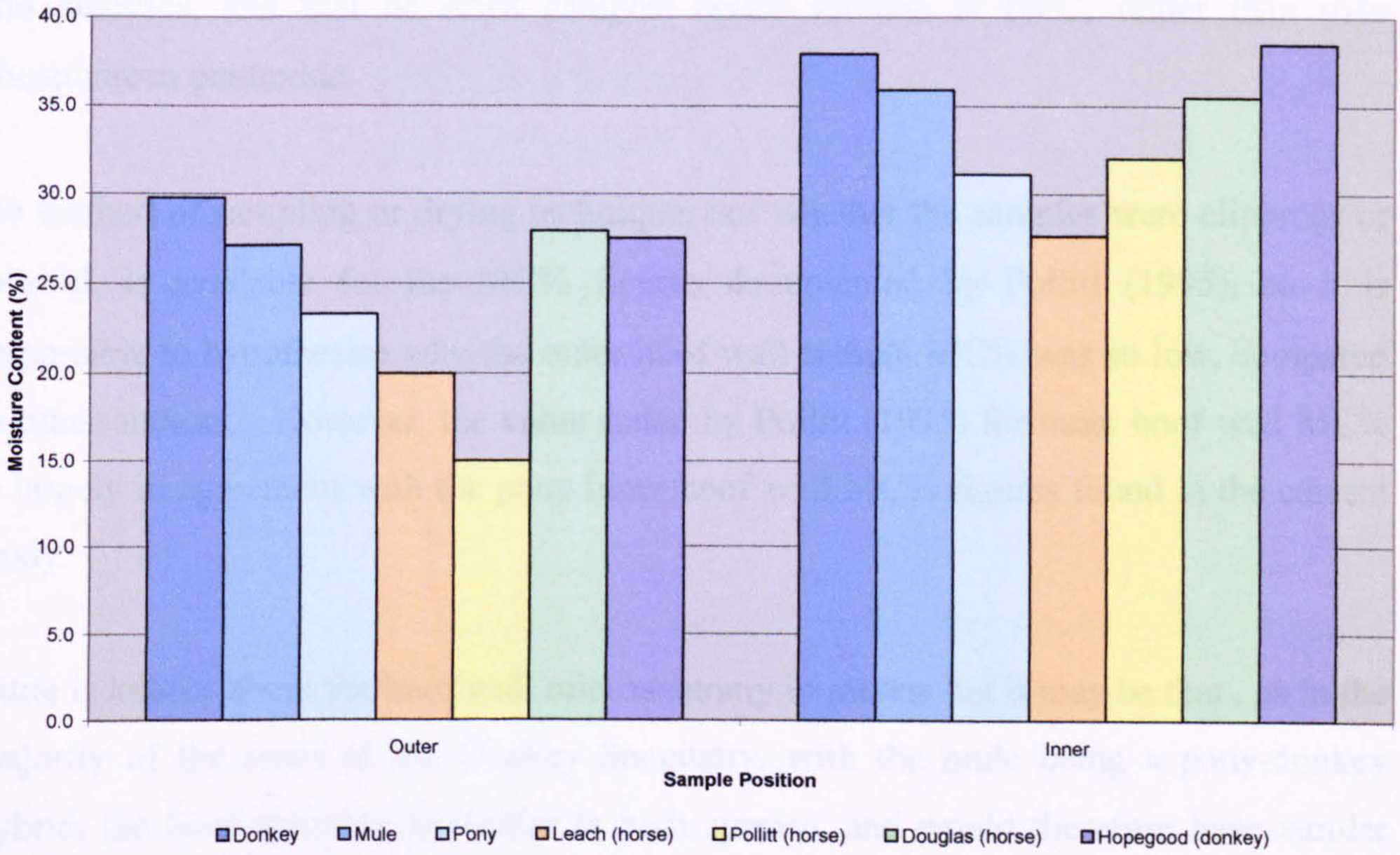


Figure 3.4.1 Comparison of the MC% of "outer" and "inner" hoof wall samples with those from previous authors

The zonal MC% of zones 1 and 2, and zones 3 and 4 were averaged to provide "outer" and "inner" hoof wall MC% measurements for all three species. These were then compared to results from previous authors (Figure 3.4.1). It has to be acknowledged that, with the exception of the Hopegood (2002) data, these comparisons are approximations, as previous authors have used different drying protocols (see Table 1.3.1).

The outer wall MC% of donkey and mule hoof in the present study was higher than that reported for any previous author, including the donkey outer wall MC% reported by Hopegood (2002). The results are similar for inner wall hoof MC%, but the donkey inner hoof wall figures are almost identical, with 38.6% in the present study and 38.5% reported by Hopegood (2002).

The low horse hoof MC% results obtained by Leach (1980) may be due to the fact that he included morbid samples in his analysis, which may have lost moisture between death and analyses, and that he dried samples under vacuum at 60°C, rather than over phosphorous pentoxide.

No method of sampling or drying technique, nor whether the samples were clippings or morbid, is available for the MC% figures documented by Pollitt (1995), so it is impossible to hypothesise why the outer hoof wall sample MC% was so low, compared to other authors. However, the value stated by Pollitt (1995) for inner hoof wall MC% is largely in agreement with the pony inner hoof wall MC% figures found in the current study.

Little is known about the hoof wall microanatomy in mules; but it may be that , as in the majority of the cases at the Donkey Sanctuary, with the mule being a pony-donkey hybrid, the hoof structure is similar to both species, and would therefore have similar moisture holding capacity. When the results for all three species are compared it is evident that both the outer and inner mule hoof MC% values are closer to those of the donkey hoof than the pony hoof (Figure 4.3.2).

Harrison *et al.*, (2007) noted that the external hoof growth rate of cattle (equivalent to zone 1 in the present study) showed significant variation to that further within the hoof wall (equivalent to zone 4 in the present study), which would indicate implications for studies such as the present one, when comparing characteristics of the zones across the hoof wall depth. A trial, similar to Harrison *et al.*,'s (2007) would need to be conducted using carbon stable isotope ratios to investigate whether donkeys, mules and ponies had comparable variability in hoof growth rate, and therefore age of horn, across the depth of the hoof.

3.5 Conclusions

- There were no significant differences ($p > 0.05$) in full hoof wall depth MC% between locations around the hoof for all three species, although both medial and lateral quarters and heels had higher hoof MC% than the midline dead centre for donkeys, mules and ponies. So although this increased MC% may be a major contributory factor in higher elasticity levels in the heels, it is not a significant increase ($p > 0.05$).

- The full hoof wall depth MC% decreased, from the winter months to the summer months for all three species, and then increased once again towards the autumn. The decrease in hoof MC% was significant ($p < 0.05$) between the winter and summer months in donkeys and ponies, but not in mules. The lowest hoof MC% was seen in May in mules and donkeys, and July in ponies. There is clearly some seasonal effect on hoof MC%, be it due to a physiological, nutritional or environmental factor, or indeed a combination of factors.

- Donkeys had significantly higher ($p < 0.05$) hoof wall depth MC% than both mules and ponies for all months except July, where the difference was only significant between donkey and mule hoof MC%. Mule hoof wall depth MC% was significantly higher ($p < 0.05$) than pony from February to September. Mule hoof wall depth MC% was on average 2.55% lower than that of the donkey hoof and 5.18% higher than that of pony hoof throughout the seasons. This indicates that the moisture characteristics of mule hoof horn are more like those of donkey, rather than pony hoof, which could have implications for the management of mules in temperate environments.

- There were no significant differences ($p > 0.05$) in zonal hoof MC% between locations around the hoof for all three species. As with the full hoof wall depth MC%, there was an insignificant increase in MC% from the midline dead centre to the heels, but this was not uniform throughout the zones. Both the medial and lateral quarters and heels had higher hoof MC% than the midline dead centre for donkeys, mules and ponies in zones 1 to 3, but this was not the case for zone 4 with no particular circumferential MC% trend shown in that zone.

- As with the full hoof wall depth MC%, the zonal hoof MC% was higher in the winter months than the summer months for all three species. The decrease in zone 1 hoof MC% was significant ($p < 0.05$) between the winter and summer months in donkey hoof, but not in mule or pony hoof. In zone 2 the decrease in hoof MC% was significant ($p < 0.05$) between the winter and summer months in both donkey and pony hoof, but not in mule hoof. Significant decreases ($p < 0.05$) in zone 3 and 4 MC% were seen between the winter and summer months in the hooves of all three species. It appears that the inner hoof wall zone MC% is more sensitive to the effects of season than the outer zones, which may be due to physiological or nutritional factors, rather than being influenced solely by the external environmental humidity or precipitation.

- Donkeys had significantly higher ($p < 0.05$) zonal hoof MC% than ponies for all months for all zones. Mule hoof MC% had significantly higher ($p < 0.05$) zonal MC% than pony for all months for zones 2 and 3; and all months except February for zone 4. The differences between donkey and mule hoof zonal MC% were not as widespread and were confined to December and May. Donkey hoof had significantly higher ($p < 0.05$) zone 1 and 2 hoof MC% than mule hoof for December, and significantly higher ($p < 0.05$) zone 2, 3 and 4 hoof MC% than mule hoof for May. This comparison substantiates the argument that mule hoof horn is closer in MC% distribution within the hoof horn to that of the donkey rather than the pony.

- When the mean zonal hoof MC% was compared, it significantly increased ($p < 0.001$) in an anterior-posterior direction towards the internal hoof structures, away from the outer hoof wall for donkeys, mules and ponies. The increase in hoof MC% from external zone 1 to internal zone 4 was 12.06% for donkeys, 12.77% for mules and 11.49% for ponies. The manner in which the interzonal hoof MC% increases occurred across the depth of the hoof wall were even in the pony hoof, but donkey and mule exhibited large increases between zones 1 and 2 hoof MC%, and 2 and 3 hoof MC%, and then a smaller increase between zone 3 and 4 hoof MC%. This is another comparison which further substantiates the argument that mule hoof horn is closer in MC% composition to the hoof horn of the donkey than that of the pony.

CHAPTER 4 – THERMAL DIFFERENTIAL SCANNING CALORIMETRY ANALYSIS OF DONKEY, MULE AND PONY HOOF HORN

4.1 Introduction

Differential scanning calorimetry (DSC) is a technique used to study the thermal transitions of a material - the changes that take place in a material when it is heated or cooled. The water content of many different biological materials has been analysed by the DSC method.

Many researchers have used high temperature thermal analysis to look at water, lipids and proteins in biological materials. Van Duzee (1986) found water and lipid transitions to be generally reversible and protein denaturations to be irreversible in *stratum corneum* samples. As protein denaturation corresponded with peaks at 85 °C and 107 °C it is questionable as to whether high temperature thermal analysis is indicative of the free/bound water fraction, as water bound to proteins will be liberated if denaturation takes place before the boiling transition of water. Indeed, Baden *et al.*, (1973) used x-rays to investigate *stratum corneum*, and suggested that the native α-keratin structure of the protein in *stratum corneum* was disrupted by heating to temperatures as low as 75-90 °C; and directly attributed a transition at 85°C to α-keratin denaturation. This has important implications for interpretation and comparisons of results, as experiments carried out at different temperatures will give different results for the same materials. If results are to be compared directly with those of others, the experimental method should be duplicated with attention to detail.

As equid hoof also has a large proportion of α-keratins within its structure, this would imply that the hoof structure, and therefore water compartmentalisation, would be affected by application of high temperature DSC.

To avoid altering the protein and lipid structure by using high temperatures, it is preferable to investigate the freezing and melting point of water in the hoof horn, to ascertain the water composition within the horn structure.

By establishing the total water content and then analysing the amount of 'freezable' water in a material, it is possible to determine the 'bound and 'free' water content of that material.

4.1.1 Bound water

As discussed in Section 1.4.1, a number of authors have defined bound (unfreezable) and free (or freezable) water for a variety of biological materials.

There has been no research into the bound and free water fractions contained within equid hoof horn, and the comparison of the hoof horn results with other biological materials is often difficult due to varying techniques and protocols, and presentation of results (Table 4.1.1). Miles *et al.*, (1994) did use DSC to investigate denaturation of tendon degeneration in the horse, but the bound water content was not ascertained.

Wortmann & Deutz (1993) characterised keratins including rhinocerous horn, porcupine quill, horse hair, echidna quill and human finger nail, using High Pressure DSC, but their work concentrated on the denaturation of the keratin α-helix around 140°C, and not on bound or free water fractions.

The majority of research, using DSC to analyse bound and free water content, to date has been carried out using *stratum corneum* (Inoue *et al.*, 1986, Takenouchi *et al.*, 1986) and eye lenses (Bettelheim *et al.*, 1982, Nunnari *et al.*, 1986 and Popdimitrova & Bettelheim, 1989). Although, Mallinson *et al.*, (2006) conducted a nutritional trial and used DSC to investigate the bound water fractions in sheep claw horn. Table 4.1.1 shows the bound water content of a number of biological materials:

Table 4.1.1 Bound water content of biological materials

Year	Author	Subject	Experimental Technique	Bound mg	Bound %
1972	Walkley, K	*Stratum corneum* (Human limb) Foot Pad *Stratum corneum* (guinea pig)	Cooled to 250°K then heated 8°K/min to 290 °K	0.34g/g dry sc 0.29g/g dry sc	
1980	Aubin, M Prud'Homme, RE Pezolet, M & Caille, JP	Muscle (Giant Barnacle)	Cooled to 245°K then heated 2.5°K/min to room temperature	0.29-0.86g/g dry wt (various water contents 57-77%)	
1982	Bettelheim, FA Christian, S & Lee, LK	Eye lens (human) normal Eye lens (human) cataractous	-30°C to + 15°C 3°C/ min melting peak	n/a	32.2% 31.8%
1986	Inoue,T Tsujii,K Okamoto, K & Toda, K	*Stratum corneum* (Human)	-40°C to + 20°C 0.6°C /min melting peak	n/a	20-30%
1986	Lundgren, CH Williams, TR & Nunnari, JM	Eye lens (Avian) Eye lens (Bovine) Eye lens (Fish) Eye lens (human) Eye lens (porcine)	From 25 °C cooled at 5°C/min to -35 °C	2.29mg/mg dry wt 1.09mg/mg dry wt 1.08mg/mg dry wt 0.84mg/mg dry wt 1.51mg/mg dry wt	
1986	Nunnari, JM Williams, TR & Powell, D	Eye lens (human)	-35 °C to + 20 °C 10-20 °C /min melting peak	n/a	42.6%
1986	Takenouchi, M Suzuki, H & Tagami, H	*Stratum corneum* (human)	Cooled to 238°K then heated 5°K/min to 278 °K	5mg/100mg bound water	
1987	Furuse, M Gonda, T Inao, S Kuchiwaki, H Nagatoshi, H & Kageyama, N	Gray Matter (rat brain) Serum (rat brain)	Room temp down to -75 °C 10 °C /min freezing peak	0.44mg/mg dry wt 1.61mg/mg dry wt	11.1% 9.3%
1989	Popdimitrova, N & Bettelheim, FA	Eye lens capsule (bovine)	-30°C to room temp 3°C/min melting peak	1.25-3.25g/g dry wt	20-50%
2006	Mallinson, J Collins, SN Daniel, ZCTR Wynn, RJ Salter, AM Buttery, PJ & Reilly, JD	Sheep claw horn Control Treated	-50°C to + 15°C 10°C /min melting peak	0.484 g/g dry wt 0.558 g/g dry wt	

The bound water content is calculated from measuring the area under the peak of the DSC curve (for more details see Section 4.2.1). The temperature that corresponds to the apex of the peak is the melting temperature. An input of energy is required to the pan

with the sample in order to make the ice melt, melting is therefore an endothermic transition.

If there is no measurable peak, then all the water contained within the material must be bound, and there is no free water to freeze. Bound water content is affected by the gross moisture content of the material as well as the relative humidity of the external environment (Swarbrick & Boylan, 1997). Relative humidity, in turn, can be affected by the temperature and partial pressure of the water vapour in the surrounding atmosphere. For a given temperature and humidity, a material will gain or lose moisture until an equilibrium is reached (this is discussed in more detail in Chapter 6), this can be defined as 'equilibrium moisture content'. Any moisture which is present in excess of the equilibrium moisture content is called "free water" (Figure 4.4.1). Bound water is the water that is retained by a material in an environment of less than 100% RH, and is a function of the solid only, and not the surroundings (Swarbrick & Boylan, 1997).

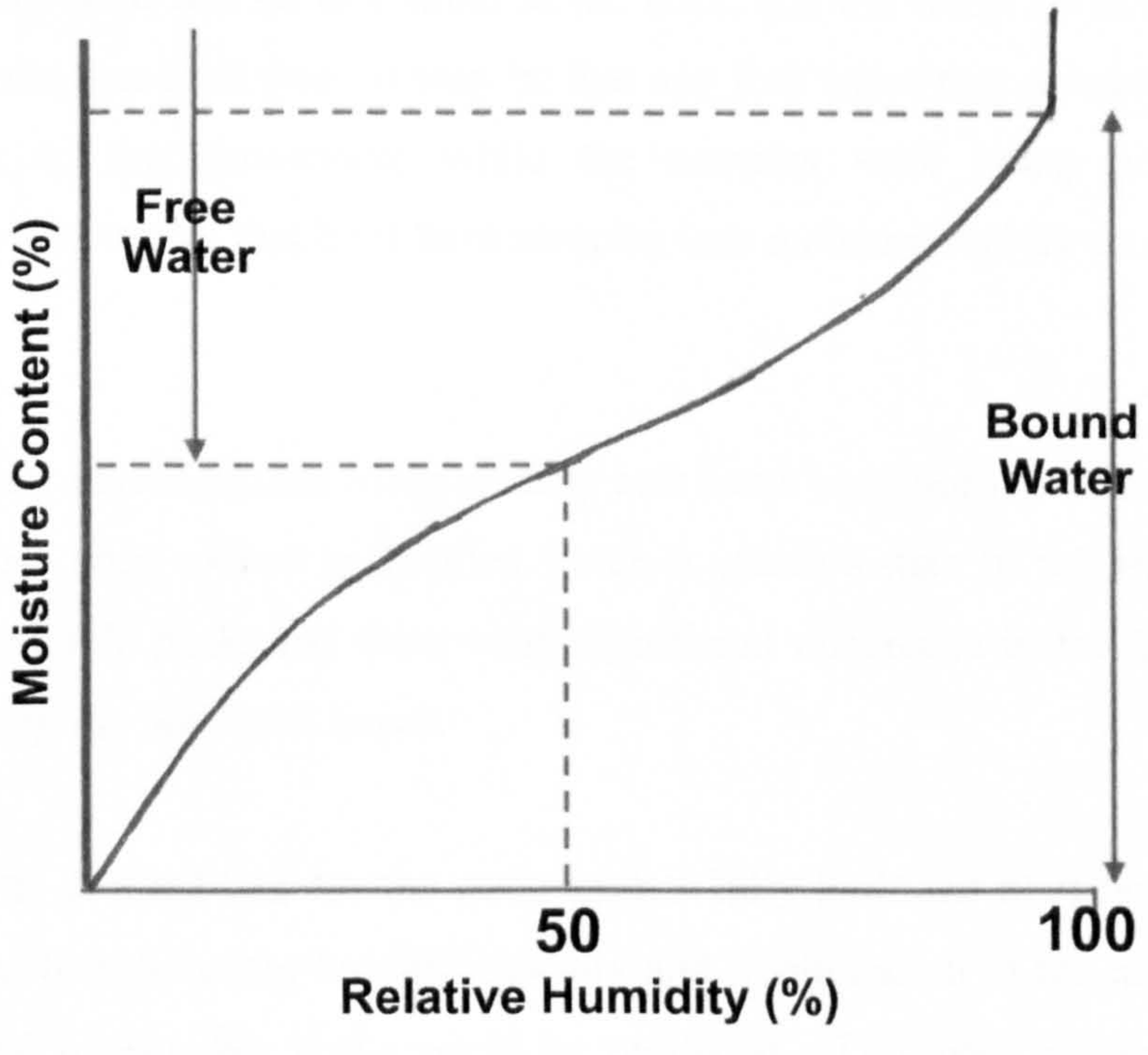


Figure 4.1.1 Relationship between bound water content, equilibrium moisture content and relative humidity for a hygroscopic solid (diagram adapted from Swarbrick & Boylan, 1997)

Takenouchi *et al.*, (1986) obtained a flat curve when they investigated *stratum corneum* with a water content of 32.7mg per 100mg dry weight; but when they looked at *stratum corneum* with water content of 38.2mg per 100mg they obtained a small peak. This would indicate that all the water contained within the 32.7mg/100mg sample was bound and unfreezable, whereas the 38.2mg/100mg sample contained free water.

Similar findings have occurred with non-biological materials. Oka (2000) found that sodium acetylhyaluronate powder which had adsorbed water, produced no peak, as the water within only exists as bound water. Once the sodium acetylhyaluronate powder was completely saturated, a peak started to appear in the DSC plots, indicating free water was present.

When fresh equid hoof material was analysed using DSC in preliminary studies, there was no discernable alteration in the base line, and no measurable peak. This is similar to the findings of Takenouchi *et al.*, (1986) looking at *stratum corneum* indicating that in the fresh state, there was no free water in the hoof, and the water content of 20-40% was all bound and non-freezable. It may be that any free water that existed in the fresh horn was lost to the atmosphere while the samples were being processed for calorimetry, as it is known that hoof horn samples lose moisture rapidly on contact with air.

Other preliminary investigations using donkey and horse hoof horn, produced evidence that horn that had been soaked in distilled water, to attain a state of full hydration, did produce a measurable peak, and there were significant difference in free/bound water fractions, between the hoof wall zones.

Therefore, it was decided that for the main study, fully hydrated hoof horn would be analysed to establish the water holding capacity and fractionation of the hoof zones, as reproducible and measurable peaks could be produced. This was in agreement with Mallinson *et al.*, (2006) who also used fully hydrated sheep claw horn when they investigated the bound water content of that material. Measurement at fully hydrated moisture content also provides a benchmark figure against which materials can be

compared. If samples are analysed at their maximal hydration, then any bound water content is the maximal value for that material at that relative humidity. This also negates any effect that the hydration status of the material may have on the bound water content.

As can be seen from Table 4.1.1, the unit that is usually used to express bound water content is mg of bound water per mg of dry matter of the material. This can be calculated using the equations in Table 4.2.10. While bound water (%) figures serve to provide an indication of what percentage of the water content of the material is bound, they do not provide an absolute measurement of the bound water content. Expressing the bound water as mg of bound water per mg of dry matter of the material means that the bound water content of samples with different moisture contents can be compared.

Various epidermal tissues have been analysed for bound water (mg/mg dry matter), including guinea pig foot pad *stratum corneum*, which contained 0.29mg/mg dry matter (Walkley, 1972). Values for human *stratum corneum* vary according to author ranging from 0.05mg/mg dry matter found by Takenouchi *et al.* (1986) to 0.34mg/mg dry matter found by Walkley (1972).

4.1.2 Aim

The aim of this part of the study was to:

- establish the bound water content within the *stratum medium* of donkey, mule and pony hoof horn at fully hydrated moisture content.

- compare the results of the three species, to establish whether there are any significant differences between donkeys, mules and ponies for *stratum medium* bound water content, at fully hydrated moisture content.

- compare the results within each species over a period of one calendar year, to establish whether there are any significant differences between months or seasons for *stratum medium* bound water content in donkey, mule or pony, at fully hydrated moisture content.

4.2 Method

4.2.1 Experimental design

The experimental design was a factorial (2x3x5), three-way mixed design, as described on page 61.

Factor 1 = Farm (Brookfield, Town Barton)
Factor 2 = Species (donkey, mule and pony)
Factor 3 = Sampling Time (December, February, May, July, September)

Bound water content of fully hydrated samples taken from hoof clippings was measured over a 12 month period in a controlled trial using donkeys, mules and ponies (Table 2.2.2), kept in mixed groups and maintained in a temperate climate in the UK. Clippings were taken every 10 weeks from the left fore hoof, and the bound water content was determined using the midline dead centre sampling site of the fully hydrated clipping (Figure 2.1.11).

4.2.2 Fully hydrated moisture content - FHMC (%)

Four zonal areas and the HWD were used as illustrated in Figure 2.1.15. All five samples from each hoof wall sampling location were then saturated in distilled water until no further mass gain was recorded to equilibrate to full hydration. This level of hydration in this study is known as fully hydrated moisture content (FHMC). The literature review provided no evidence of research into whether this level of hydration could be reached *in vivo*. However, it may be that this could be achieved in hoof horn of horses which live predominantly in marshland areas such as the Camargue breed in France (Groves, 1974).

As previous methods of hoof material hydration were not explained fully by Benedetti (1948), Bertram & Gosline (1987) or Spitzlei (1996), a preliminary experiment was

undertaken using donkey hoof saturated with distilled water, until no further mass gain was recorded. This study ascertained that FHMC was achieved within 7 days of saturation (personal observation).

After 7 days, the samples were then blotted on tissue paper to remove excess water and weighed using the Sartorius Balance, before being placed in a desiccator with phosphorous pentoxide (P_2O_5) to attain equilibrium at 0%RH for 2 weeks, until no further mass loss was recorded (Hopegood, 2002).

The samples were then reweighed and the fully hydrated moisture content (FHMC %) calculated using the following equation:

Equation 2

$$\text{FHMC \%} = \frac{\text{Wet Weight}^a - \text{Dry Weight}^b}{\text{Wet Weight}^a} \times 100$$

Key for Equation 2

[a] Wet weight = Mass of sample following full hydration

[b] Dry weight = Mass of sample following no further mass loss after drying over P_2O_5

This equation gave the fully hydrated moisture content and as it is a manipulated value, it cannot be used in comparisons with moistures calculated from fresh hoof material.

4.2.3 Differential scanning calorimetry (DSC)

DSC is a thermal analysis technique which analyses changes that take place in a material when it is heated or cooled, such as the melting or freezing of water. Analysis of the melting and freezing transitions of water enables determination of the free water fraction, as bound water does not freeze at 0°C (Lundgren *et al.*, 1986). Lui & Yao (2001) stated that unfrozen bound water plays an important part in biological systems and contributes favourably to the conformational stability of proteins.

When a sample is heated and the melting temperature of ice is reached, the hydrogen bonds in the water crystals break up and the ice melts; in order to do this, the crystals must absorb heat. Melting is a first order transition (Alcoutlabi & McKenna1, 2005). Melting is a thermal transition that involves both a latent heat, which is the heat absorbed or emitted by a sample during an isothermal phase transition, where there is no temperature change, and a change in the heat capacity of the material. Thus, when the melting temperature is reached, the sample temperature will not rise until all the crystals have melted. The heater under the sample pan has to transfer a considerable amount of heat into the sample in order to both melt the ice crystals and keep the temperature rising at the same rate as that of the reference pan. This extra heat produced by the heater under the sample pan during melting is represented by a peak on the DSC plot (Figures 4.2.7 and 4.2.8).

Samples were prepared according to the hoof sampling methods described previously in Section 2.1.1, and were stored at 5°C in distilled water for at least 7 days to equilibrate to full hydration (100%). The samples were processed using a Perkin Elmer DSC4 (PerkinElmer, Inc., USA), with a 7mg aluminium blank reference pan.

The sample pan was weighed and zeroed on the Sartorius microgram balance with an uncertainty no greater than ±0.010 mg. Then a hoof sample of approximately 10-15mg was removed from distilled water storage, blotted on a paper towel to remove excess surface water and weighed in the pan (Figure 4.2.1).

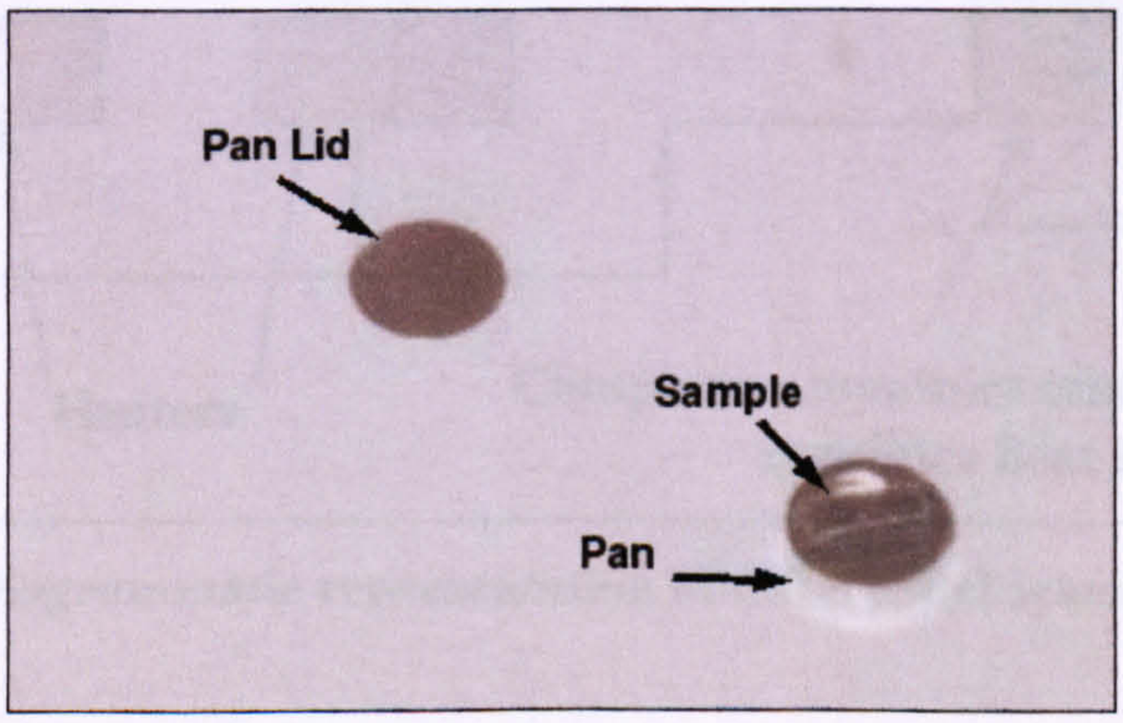


Figure 4.2.1 Sample in DSC pan prior to crimping

A sample pan lid was then placed quickly on top of the sample, and the edges of the pan were crimped, using a Perkin Elmer crimper, to avoid moisture loss (Figures 4.2.2 and 4.2.3).

Figure 4.2.2 Perkin Elmer DSC crimper

Figure 4.2.3 Sealed DSC pan containing sample

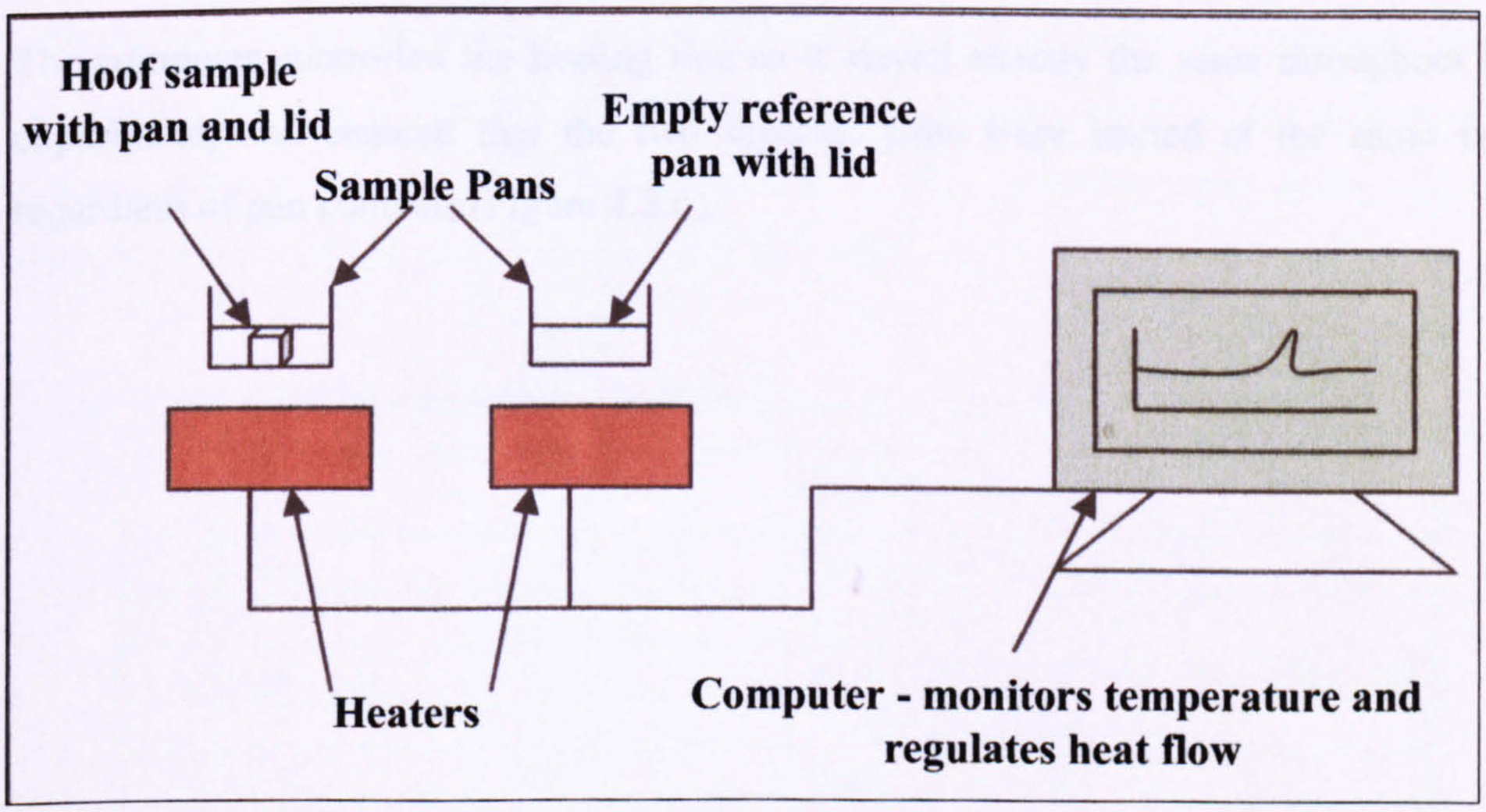


Figure 4.2.4 Diagrammatic representation of differential scanning calorimeter

The apparatus had two pans (Figures 4.2.4 and 4.2.5), one contained the sample and the other was left empty as a reference pan. The platinum covers were placed over each pan

using forceps. The head of the DSC was then closed. The head was maintained at the start temperature by pulsed nitrogen. Each pan sat on top of a heater; the computer then turned on the heaters, and regulated the heating of the two pans at a specific rate. In this study the rate of cooling/heating was 10°C per minute.

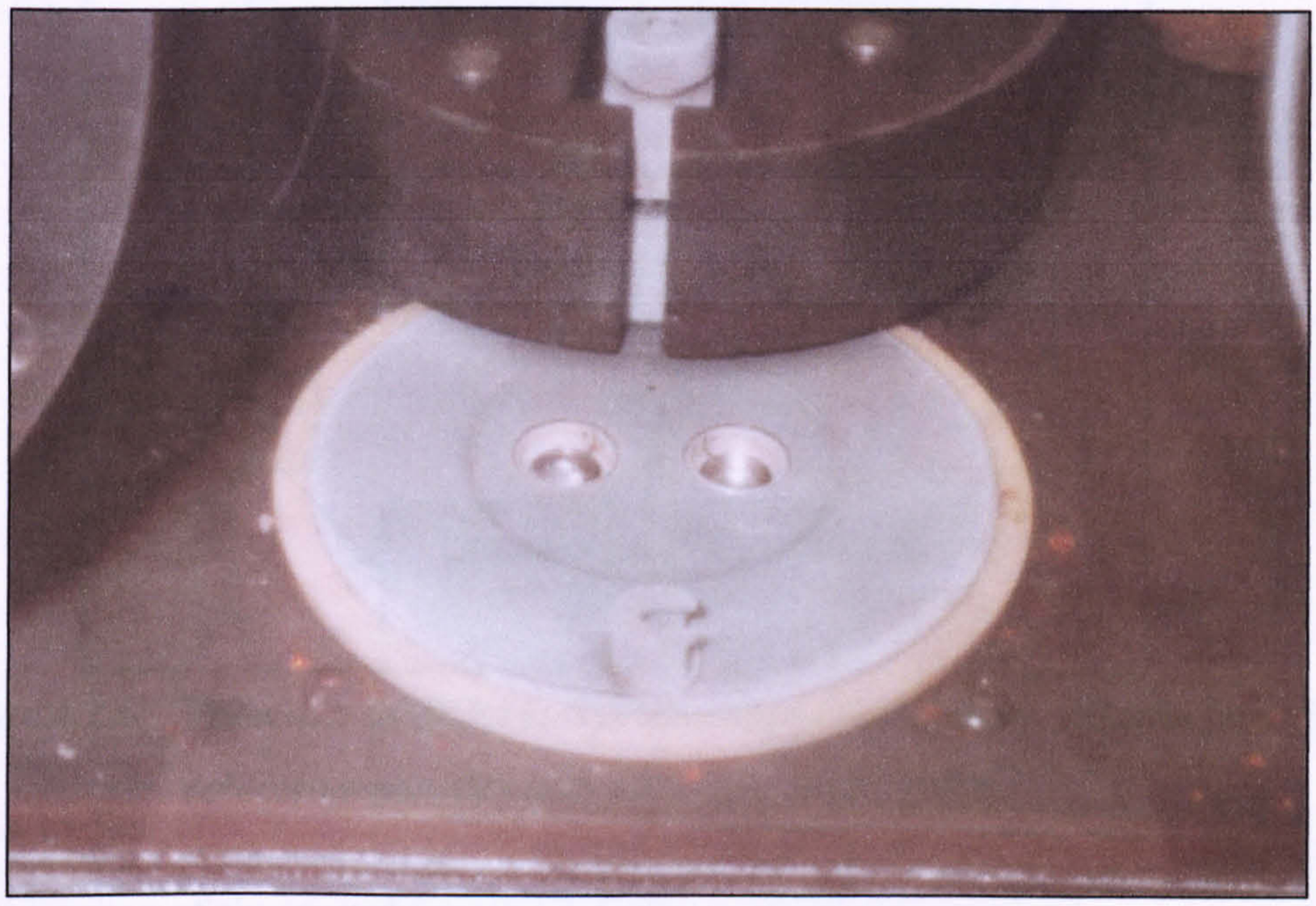


Figure 4.2.5 DSC head with protective cover removed, showing sample pans

The computer controlled the heating rate so it stayed exactly the same throughout the experiment, and ensured that the two separate pans were heated at the same rate, regardless of pan contents (Figure 4.2.6).

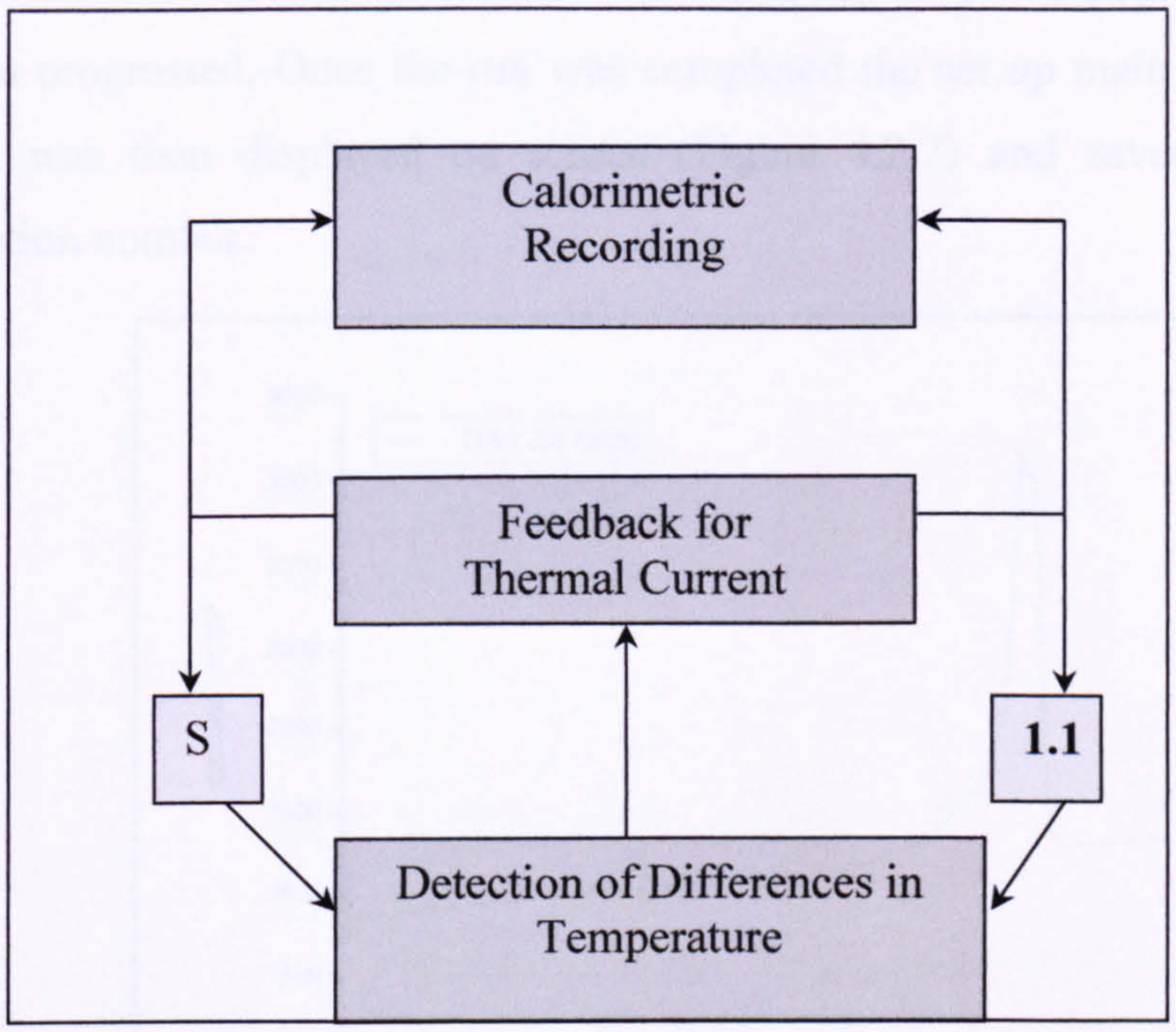


Figure 4.2.6 Principle of differential scanning calorimetry, sample pan (S) and reference pan (R) (after Furuse *et al.*, 1987)

The presence of the sample material in the sample pan required more energy input to keep the temperature of that pan increasing at the same rate as that of the reference pan. The heater underneath the sample pan had to increase heat output (mcal/second) over and above the rate of heating of the reference pan (Figure 4.2.4). This difference in heater output was what was measured by DSC. The following information was then entered into the computer to program the DSC cooling run:

Table 4.2.1 DSC programming information - cooling run

Parameter	Figure	Explanation
T FINAL	-50	Bottom of plot scale (^{o}C)
T MIN	-50	Lowest Temperature (^{o}C)
T INCREMENT	6.5	Temperature change divisible by 10 this gives the scale
T INITIAL	15	Start Temperature (^{o}C)
Y RANGE	10	Preset
HEATING RATE	10	Degrees per minute (^{o}C)
COOLING RATE	10	Degrees per minute (^{o}C)
SAMPLE WEIGHT	10	Weight from balance in mg

Once the DSC cooling run was started by pressing the trace was displayed on the screen as the run progressed. Once the run was completed the set up main menu reappeared. The plot was then displayed on screen (Figure 4.2.7) and saved using a unique identification number.

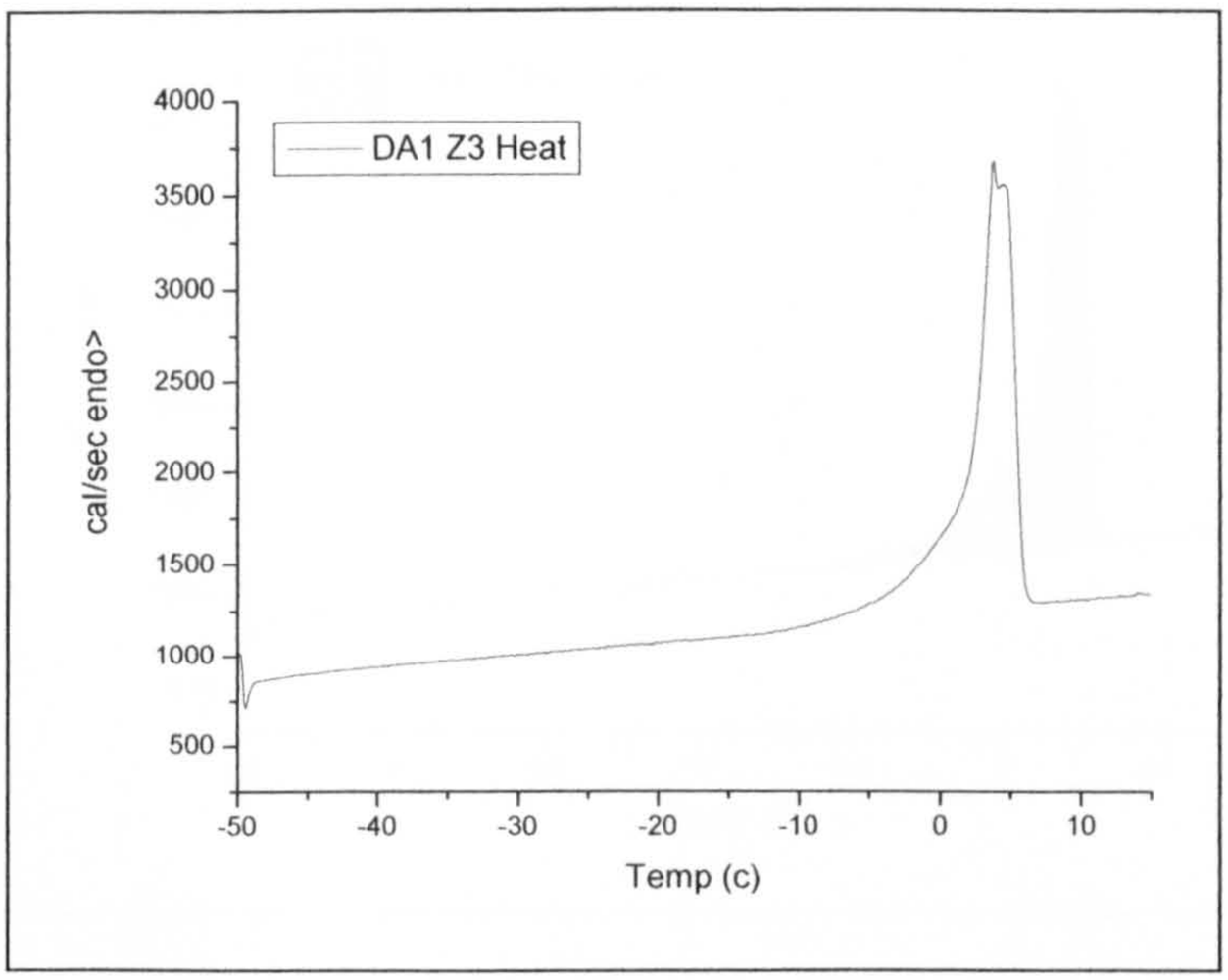


Figure 4.2.7 Graphical representation of DSC screen showing a completed run with peak

Once the plot was saved the following information was then entered into the computer to program the DSC heating run:

Table 4.2.2 DSC programming information - heating run

Parameter	Figure	Explanation
T FINAL	15	Top of plot scale (^{o}C)
T MIN	-50	Lowest Temperature (^{o}C)
T INCREMENT	6.5	Temperature change divisible by 10 – gives scale
T INITIAL	-50	Start Temperature (^{o}C)
Y RANGE	10	Preset
HEATING RATE	10	Degrees per minute (^{o}C)
COOLING RATE	10	Degrees per minute (^{o}C)
SAMPLE WEIGHT	10	Weight from balance in mg

Again, once the run was completed the set-up main menu reappeared and the plot could be saved.

As the temperature increased (or decreased) on a typical DSC result the x-axis was the temperature, and the y-axis was the difference in heat output of the two heaters at a given temperature (Figure 4.2.7).

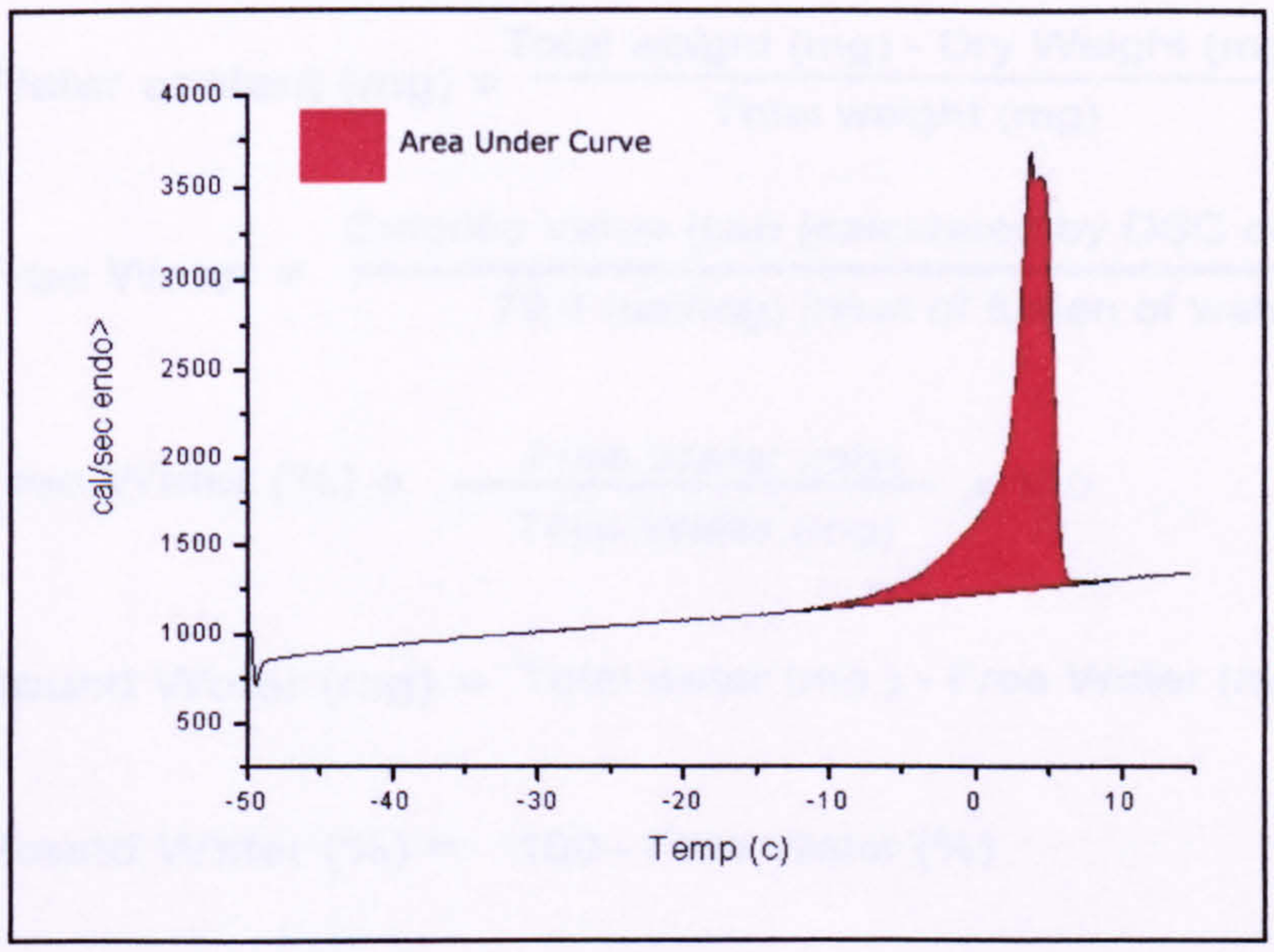


Figure 4.2.8 Determination of area under curve

Melting phenomena appear as peaks, and thus the area under the curve could be calculated. Once a baseline had been fitted to the curve, it was possible to calculate the area under the curve (Figure 4.2.8) in units of calories per gram (the weight of the sample being entered at the beginning of the heating/cooling run) by using the DSC computer.

This meant that the area under the curve was expressed as calories released (or absorbed) per gram of sample, as weighed into the DSC pan prior to heating or cooling. This figure was then used to calculate the bound water fraction contained within the sample (Figure 4.2.9). The sample dry weight was determined by drying a duplicate sample, taken adjacent at the same time as the DSC sample, from the same hoof wall zone as the DSC sample, in a desiccator containing P_2O_5 (see section 3.2.2).

Equation 3 $$\text{Total Water (mg)} = \text{Total weight (mg) - Dry Weight (mg)}$$

Equation 4 $$\text{Water content (mg)} = \frac{\text{Total weight (mg) - Dry Weight (mg)}}{\text{Total weight (mg)}} \times 100$$

Equation 5 $$\text{Free Water} = \frac{\text{Calorific Value (cal) (calculated by DSC computer)}}{\text{79.4 (cal/mg) [heat of fusion of water]}}$$

Equation 6 $$\text{Free Water (\%)} = \frac{\text{Free Water (mg)}}{\text{Total Water (mg)}} \times 100$$

Equation 7 $$\text{Bound Water (mg)} = \text{Total water (mg) - Free Water (mg)}$$

Equation 8 $$\text{Bound Water (\%)} = \text{100 - Free Water (\%)}$$

Equation 9 $$\text{Water binding to solid tissue component} = \frac{\text{Bound Water (mg)}}{\text{Dry Weight (mg)}}$$

Figure 4.2.9 Calculation of water components (after Furuse *et al.*, 1987)

Although the equation in Figure 4.2.9 can be used to calculate the free and bound proportions of water contained within the sample, it does not give a precise value for the bound water contained within the sample. It gives the percentage of the total water content that is composed of bound water rather than an absolute value. As can be seen from Table 4.1.1, a value that is also used is mg of bound water per mg of dry matter (of the sample). This was calculated using the equations in Figure 4.2.10.

Equation 10 Total Water (mg) = Moisture content x Wet Sample (mg)

Equation 11 Dry Weight (mg) = Wet Weight (mg) - Total Water (mg)

Equation 12 $$\text{Water per 100mg dry weight} = \frac{100}{\text{Dry Weight (mg)}} \times \text{Total Water (mg)}$$

Equation 13 Bound Water (mg/100mg dry weight) = Water per 100mg Dry Weight x Bound Water

Figure 4.2.10 Calculations to obtain bound water (mg/100mg dry weight)

4.2.4 Statistical analyses

All data were analysed using SPSS 14 for Windows (SPSS Inc., Chicago, Illinois 60606). The data sets were checked for normality using normal probability plots. Data was evaluated for reduction using Factor Analysis and then General Linear Models were used to analyse the reduced data set. Significance for all analyses was assessed at a minimum of $p < 0.05$.

4.3 Differential scanning calorimetry results

4.3.1 Overview

These results need to be treated with caution due to the paucity of data for the full hoof wall depth BWC analyses. While the technique of using the midline dead centre sampling site is easily reproducible in each animal, it provided variable results. Particularly as donkey hoof wear lead to intermittent sampling of complete clippings and so too many values were lost, predominantly from the midline dead centre hoof sampling location. However, from the results which were obtained, it was possible to see that there were differences in hoof wall BWC (mg/mg dry matter) both between species and between zones within the hooves of each species.

The overall trend in all species was an increase in the amount of bound water (mg/mg dry matter) from the outer hoof wall in zone 1 to the inner aspect of the *stratum internum* at zone 4. Zones 1 and 2 had lower BWC (mg/mg dry matter) than zones 3 and 4 for donkey and mule on all sampling dates. Pony BWC (mg/mg dry matter) while normally lower than both donkey and mule, was a little more variable, with values for zones 1 & 2 in May being higher than both donkey and mule.

The data is presented in the following order, for logical presentation and to extract all different comparisons:

4.3.2 General linear model analysis of the effects of species, farm, month, gender and pigment on full hoof wall depth BWC

Mixed between-within subjects analyses of variance were attempted on the full hoof wall depth BWC results, to try to assess the impact of farm, sex and species on hoof BWC across the calendar year (December – September sampling occasions). It was not possible to produce multivariate test statistics as there were insufficient degrees of freedom (Table 4.3.1) due to the paucity of data. Only one donkey, two mules and three ponies had BWC data for all the sampling dates.

Table 4.3.1 Full hoof wall depth hoof BWC for donkeys, mules and ponies across the calendar year

Time Period	Donkey			Mule			Pony		
	n	Mean	$\pm SE_D$	n	Mean	$\pm SE_D$	n	Mean	$\pm SE_D$
December	1	0.957	-	2	0.480	0.171	3	0.456	0.148
February	1	0.749	-	2	0.546	0.018	3	0.442	0.072
May	1	0.621	-	2	0.501	0.017	3	0.422	0.034
July	1	0.579	-	2	0.486	0.003	3	0.412	0.010
September	1	0.715	-	2	0.501	0.033	3	0.440	0.030

Due to the lack of data available, any inferential statistical analysis of the full hoof wall depth BWC results is unfeasible. Had sufficient data been available, a full general linear model analysis of the results would have been performed.

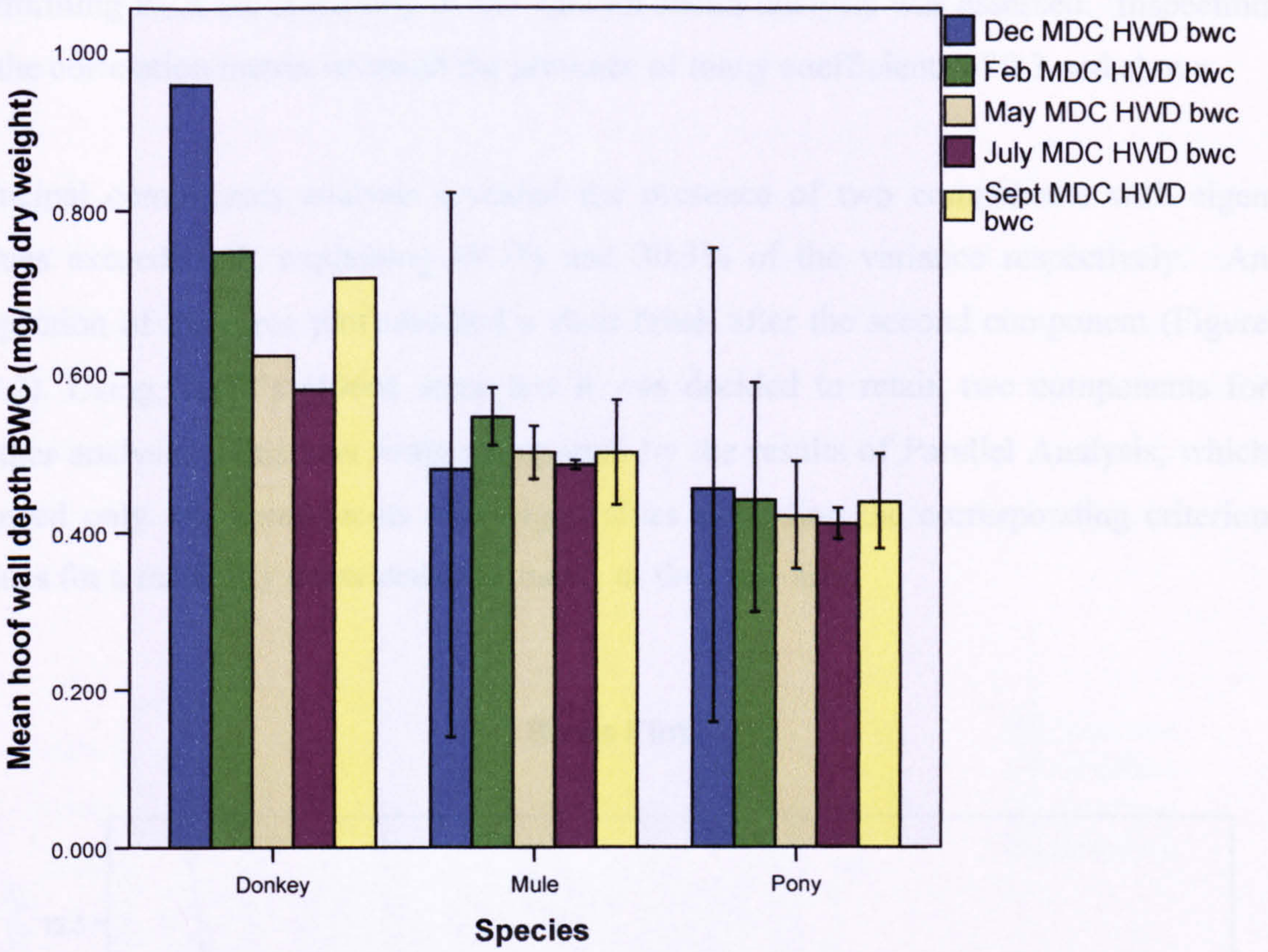


Figure 4.3.1 Mean hoof wall depth BWC (mg/mg dry weight) of each species through the calendar year

Figure 4.3.1 shows that the hoof wall depth BWC (mg/mg dry weight) varied over the calendar year, with a general trend to decrease in the summer months for all species. An initial assumption could be that donkey hoof appears to contain almost twice as much bound water as pony hoof. Donkey hoof also seems to have the most variation in BWC throughout the calendar year, although interpretation of these results has to be guarded due to the low sample numbers.

4.3.3 Factor analysis of zonal BWC

Factor analysis was conducted in order to reduce the zonal BWC data set. Analyses were carried out on the zonal data for each month. The zonal BWC results were subjected to principal components analysis (PCA) using SPSS version 14. Prior to

performing PCA the suitability of the data for factor analysis was assessed. Inspection of the correlation matrix revealed the presence of many coefficients of 0.3 and above.

Principal components analysis revealed the presence of two components with eigen values exceeding 1, explaining 69.7% and 30.3% of the variance respectively. An inspection of the scree plot revealed a clear break after the second component (Figure 4.3.2). Using Catell's (1966) scree test it was decided to retain two components for further analysis. This was further supported by the results of Parallel Analysis, which showed only two components with eigenvalues exceeding the corresponding criterion values for a randomly generated data matrix of the same size.

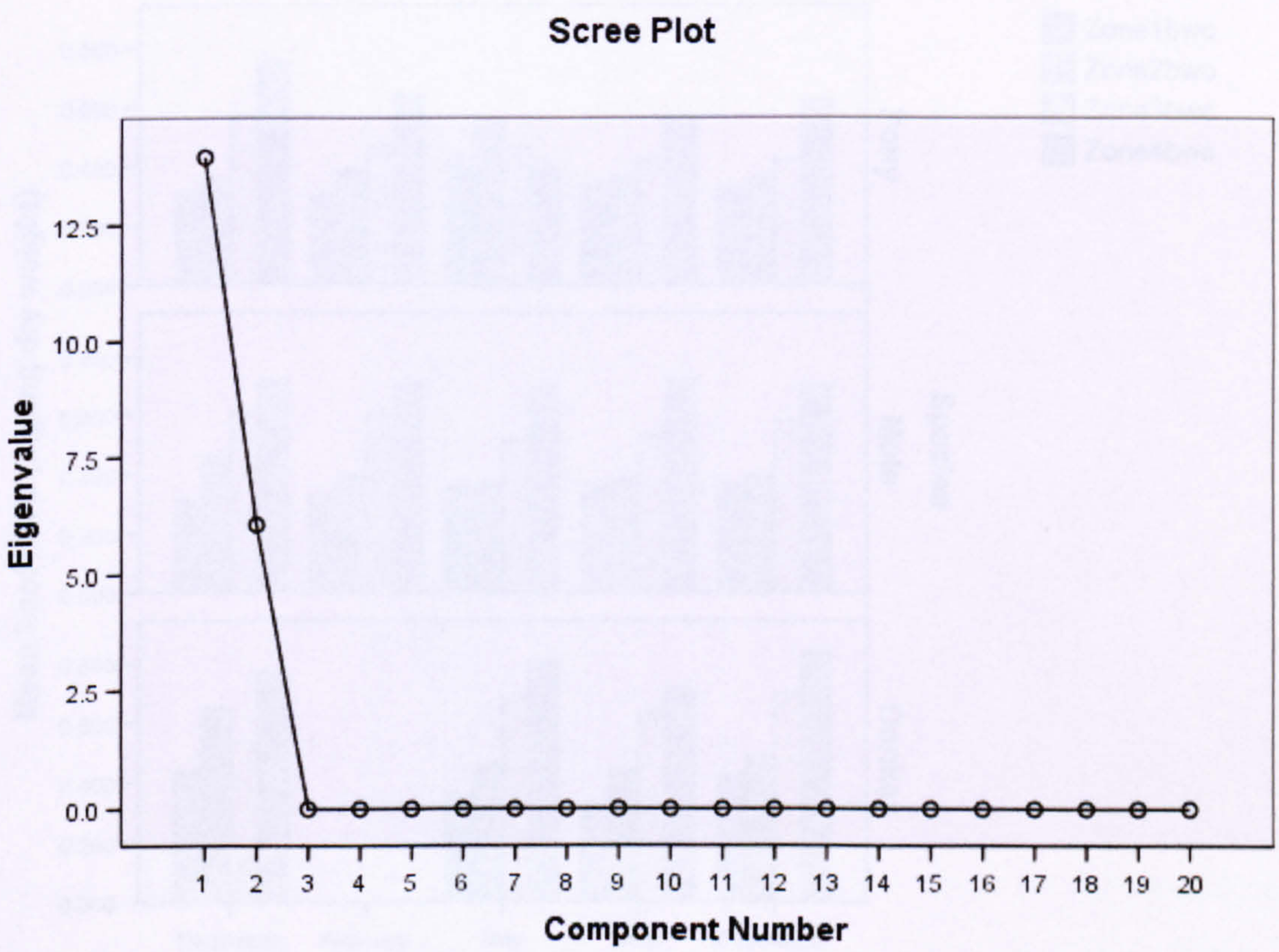


Figure 4.3.2 Scree test plot of BWC of hoof wall zonal samples

It was decided that, as the two factors loaded on all the variables, all the BWC results would be meaned to reduce the number of variables.

4.3.4 General linear model analysis of the effects of species, farm, month, gender and pigment on zonal hoof BWC

Mixed between-within subjects analyses of variance were attempted on the zonal hoof wall BWC results, to try to assess the impact of farm, sex and species on zonal hoof BWC across the calendar year (December – September sampling occasions). As with the full hoof wall depth analyses, it was not possible to produce multivariate test statistics as there were insufficient degrees of freedom due to the lack of data for donkey hoof in February (Figure 4.3.3).

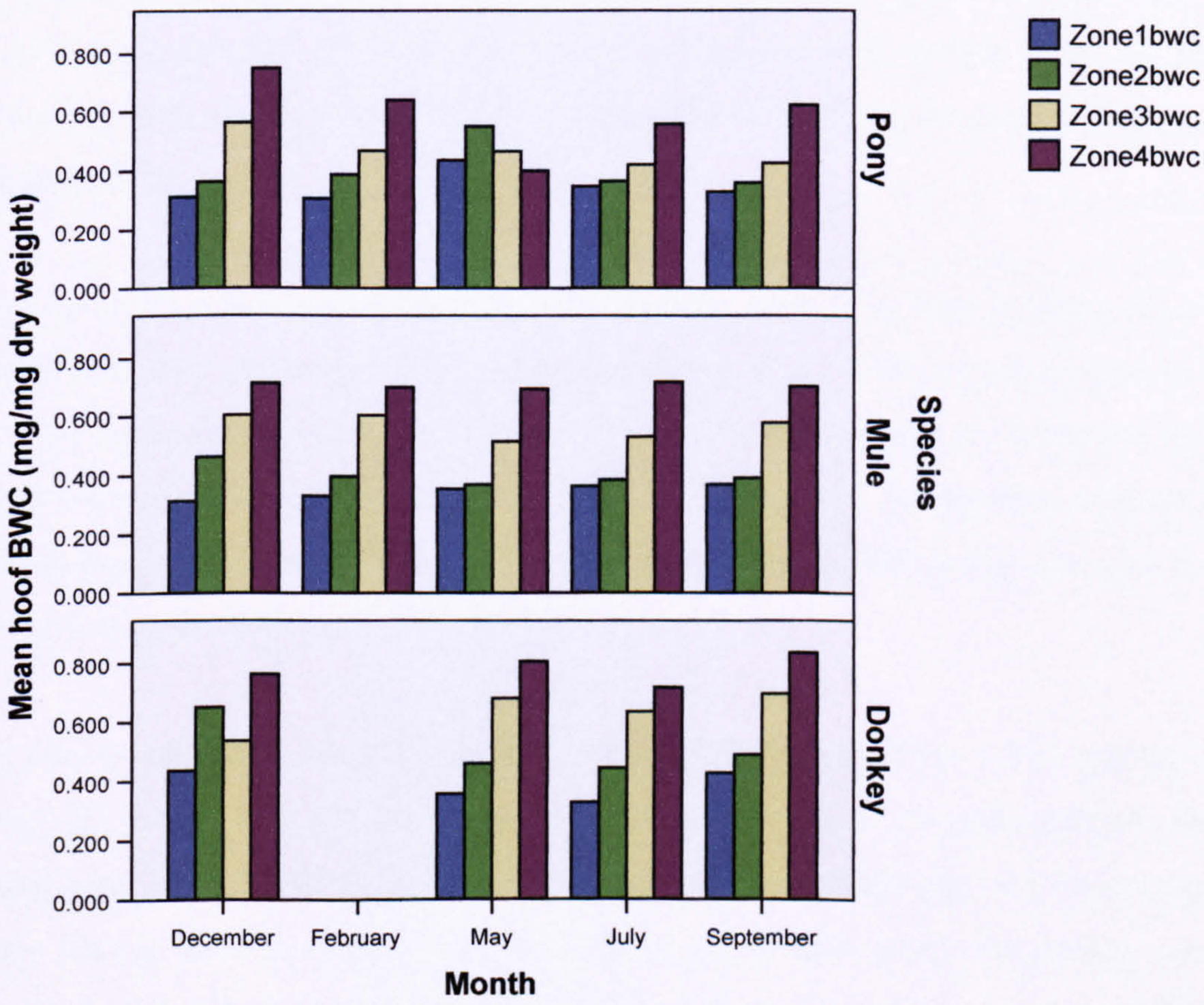


Figure 4.3.3 Mean hoof wall zonal BWC (mg/mg dry weight) of each species through the calendar year

Figure 4.3.3 shows that the zonal hoof BWC (mg/mg dry weight) did not appear to vary as much over the calendar year as the full hoof wall depth BWC, although the pony BWC in May clearly reverses the general trend for the pony BWC in the other months, for all zones. Inter-species variation in hoof BWC is evident with donkey hoof having a higher BWC than pony for most zones in most months. Again, it must be stressed that interpretation of these results has to be guarded due to the low sample numbers.

4.4 Discussion

Overall, the donkeys had the highest hoof BWC (mg/mg weight dry matter) and the ponies had the lowest. For each of the species, the lowest full hoof wall depth BWC was in the months of May and July (Figure 4.3.1) - these were also the driest and warmest months, and the animals were turned out at pasture during this period. Mules also appeared to have low full hoof wall depth BWC in December.

Takenouchi *et al.* (1986) found figures of 0.05 mg/mg dry weight BWC for human *stratum corneum*. The hoof wall *stratum medium* mg/mg dry weight BWC figures for donkey, mule and pony are considerably higher than this; with the lowest figure being 0.208mg/mg (±0.026 SE_D) for the zone 1 pony hoof wall sample from the February sampling date, and the highest 0.807mg/mg (±0.015 SE_D) for the zone 4 donkey sample taken in May.

The figure of 0.807mg/mg dry matter for donkey zone 4 in May is fairly close to that found in human eye lens at 0.84mg/mg (Lundgren *et al.,* 1986) or giant barnacle muscle at 0.86mg/mg dry weight (Aubin *et al.,* 1980). These structures are known to have high moisture contents (57-77%) and to be capable of extensive deformation, a characteristic which has been demonstrated by Bertram & Gosline (1987) using material from the inner hoof wall of the horse.

As may be expected, the cross-species zonal BWC figures, from 0.361 mg/mg (±0.064 SE_D) for zone 1 to 0.668 mg/mg (±0.133 SE_D) for zone 4, are comparable to those for sheep claw horn (Mallinson *et al.,* 2006). The value of 0.484 g/g DM for control sheep claw horn is closest to the value reported in the present study for donkey zone 2 in September (0.488 mg/mg ±0.038 SE_D). The 0.558 g/g DM sheep claw horn BWC of the treated animals in Mallinson *et al.*,'s (2006) study is very close to the 0.557 mg/mg (±0.072 SE_D) BWC found in pony hoof zone 4 in May.

Lui & Yao (2001) stated that bound water plays an important part in biological systems, and contributes favourably to the conformational stability of proteins. This would

indicate that the BWC gradient across the hoof wall depth is vital to structure and function of the hoof wall zones of all the species studied, where keratin of the primary structural protein involved. Zone 4, which is never less than 0.717mg/mg (±0.038 SE_D) dry weight BWC in donkeys, 0.696mg/mg (±0.032 SE_D) dry weight BWC in mules, and never less than 0.400mg/mg (±0.111 SE_D) dry weight BWC in ponies, not only contains high levels of bound water, but also has a predominantly high moisture content (%) overall. These high levels of BWC would suggest that, not only is bound water fundamental to the conformational stability of proteins, but it also by virtue of this it primarily influences the deformational characteristics of the hoof horn, which are so vital to the function of the hoof.

The pony hoof wall BWC showed a gradual increase in BWC from zone 1 to zone 3 with interzonal increases of 0.059 mg/mg (±0.009 SE_D) between zones 1 and 2 and 0.044 mg/mg (±0.019 SE_D) between zones 2 and 3, and then a larger increase of 0.116 mg/mg (±0.022 SE_D) between zones 3 and 4. The mule hoof BWC increase was similar to that of the ponies between zones 1 and 2 (0.050 mg/mg ±0.013 SE_D), but then showed distinct divisions with larger increases between zones 2 and 3 (0.168 mg/mg ±0.015 SE_D) and zones 3 and 4 (0.139 mg/mg ±0.023 SE_D). The interzonal increases in BWC across the zones of donkey hoof were different to both pony and mule, with large increases in BWC between zones 1 and 2 (0.121 mg/mg ±0.021 SE_D), zones 2 and 3 (0.147 mg/mg ±0.027 SE_D) and also a large increase of 0.132 mg/mg (±0.028 SE_D) between zones 3 and 4.

This comparison contradicts the interspecies findings for the moisture content analyses, where mule and donkey hoof were very similar in their zonal moisture content gradient across the entire hoof wall (Table 3.3.11). The bound water content increases between zones 2 and 4 are indeed similar for the two species, but the increases between zones 1 and 2 are markedly different for donkey (0.121 mg/mg ±0.021 SE_D) and mule (0.050 mg/mg ±0.013 SE_D). The interzonal increase between zones 1 and 2 for mule is closer to that for the pony 0.059 mg/mg (±0.009 SE_D). This may indicate that the water binding characteristics of the mule hoof are some kind of hybrid between those of the pony between zones 1 and 2, and the donkey between zones 2 and 4. Further

investigation of the water binding capacity of the hoof horn of all three species is needed to establish whether this hypothesis is valid.

As discussed in section 4.1.1, the level of free water within the hoof structure is affected by external as well as internal factors. A lower relative humidity (%) in the external environment would cause the hoof to lose water to the environment. By its very nature, the free water is lost first, followed by that bound water that converts to free at that EMC (Figure 4.1.1). Imokawa *et al.*, (1991) described bound water to be held within the multilipid bilayers of the *stratum corneum*, and referred to a general resistance against free water flux provided by the water permeability barrier which has been described by others as being a 3-5 cell deep layer in equine hoof (Kempson and Campbell 1998). Imokowa *et al.*, (1991) stated that bound water could be extracted with *stratum corneum* lipids and replaced when the *stratum corneum* lipids were reinstated. As it is known that in the final step in the cornification of the hoof wall, the keratinocytes secrete lipids into the extracellular space (Grosenbaugh & Hood, 1993), it could be assumed that the hoof has similar mechanisms for storing bound water.

Similarly, Mallinson *et al.*, (2006) found that an increase in the gross and bound water content of the hoof was closely linked with an increase in lipids within the hoof, which were administered in a feed supplement. They hypothesised that the lipid supplementation may result in permanent changes to the sheep horn, as bound water content is less labile and subject to extraction from the horn, than free water. Mallinson *et al.*, (2006) also suggested that this may lead to increased flexibility of the claw horn, leading to increased absorption of loads imposed through locomotion. In another nutritional trial, this time in pigs, Simmins (1985) also found a correlation between improved claw integrity and increased fatty acid levels in the hoof horn of pigs fed high level biotin diets. The results from these studies indicate that further research, using donkeys, mules and ponies as the species subjects, would be of interest to ascertain whether bound water, lipid profiles and dietary intake are interrelated in these species.

If the hoof was submerged in water, as when the animal is grazing in rain or dew soaked grass, or is hosed for long periods, water would be absorbed through the outer hoof wall,

as hoof horn is hygroscopic. This gain or loss of water would have consequence for the locomotion of the animal, as keratin is plasticised by water, and so hoof moisture content affects the mechanical properties of the hoof wall (Hopegood., 2002).

Hoof water loss would be more critical in an environment of low relative humidity. Lui and Yao (2001) have indicated that water lost from the bound fraction could have an effect on the conformational stability of the protein, and this could be so within zone 1 of the hoof wall. So not only would the hoof wall become stiff through free water loss, it would also be compromised by the instability of the proteins if bound water was also lost. This would be detrimental to the function of the hoof, and may be a contributing factor to the zone 1 wall delamination (Figure 1.2.12) that Thomason *et al.*, (1992) attributed the hydration gradients within the hoof material. Even if a small proportion of the bound water contained in zone 1 was to be lost, it would result in a reduction in the plasticity of the hoof wall, and therefore an increase in the brittleness of the material, leading to fracture. This would possibly be more critical for mules as they have the lowest mean BWC in zone 1, of 0.348 mg/mg (±0.035 SE_D) dry weight.

All species show an increase in the BWC towards the inner *stratum medium* zones, which is understandable given that the labile moisture from the laminar tissues is gradually transferred through the hoof wall tissue to the outer surface (Pollitt, 1998). The average rise in hoof BWC (mg/mg dry matter) from external zone 1 to internal zone 4 was 0.400 mg/mg for donkeys, 0.357 mg/mg for mules and 0.219 mg/mg for ponies. This would indicate that there is a greater amount of bound water contained within the donkey hoof, which could be transferred from the inner hoof zones to the outer zones, should the environmental conditions dictate that it was required. This would benefit animals in an environment of low relative humidity, which is the natural environment of the donkey, and so may be an evolutional adaptation of the species.

Kasapi & Gosline (1998) investigated gross hoof wall hydration in horse hoof horn in vitro by suspending cubes of horn in a relative humidity environment of 97% within a hydration/dehydration chamber. Specimens were coated with wax on all but one side and suspended in the chamber. They found that the hydration rate was greater from the

inner *stratum medium* surface than the outer *stratum externum*. A similar experiment could be repeated with the addition of 'snapshot' samples being taken for bound water analysis at specified time intervals, although care would need to be taken to maintain the relative humidity environment right up to insertion of the samples into the DSC machine so that no moisture was lost in sample processing.

Although inferential statistical analyses of any possible seasonal effect on the bound water content of the hoof was impossible, the descriptive statistics (Appendix 2) appear to indicate that there is little variation in the zone 1 hoof BWC of all species between months. The zone 1 BWC ranges from 0.308mg/mg (±0.026 SE_D) dry matter for ponies in February up to 0.436mg/mg (±0.040 SE_D) dry matter for donkeys in December. The inner zones 3 and 4 in mule and pony hoof horn appear to have lower hoof BWC in the summer months, whereas the donkey zone 3 and 4 hoof BWC increases in the summer months. It is unclear as to why these zonal and species phenomena may occur.

Butler (1992) suggested that the hoof tubules were hollow to conduct water and facilitate hydration of distal portions of the wall, however no hydration gradient has been found to substantiate this. Indeed, Leach (1980) and Hopegood (2002) found a decreasing gradient extending distally from the coronary band. In support of this, Kasapi & Gosline (1998) found that tubules do not facilitate the conduction of water down the hoof wall. So it appears that the tubules themselves are not involved in the transport of gross water within the hoof wall. Further research is required to investigate whether they are involved in the hydration of the wall through their bound water content. For instance, the samples used in the present study were taken from the distal edge of the bearing border, and any comparison with 50% hoof wall height samples has to be aired with caution due to the presence of this vertical hydration gradient within the hoof until the presence of any possible vertical bound water content gradient has been confirmed or refuted.

It may be that the intertubular horn fraction of the *stratum medium* is related to the BWC of the hoof material. It would be prudent when undertaking any further work into the relationship between the hoof wall depth and bound water content (mg/mg dry matter) to

also take account of tubule morphology and the proportion of intertubular horn within the hoof material.

As mentioned in Section 1.2.9, intertubular horn comprises of keratinocytes which have matured between the hoof wall tubules and forms a matrix between them (Nickel, 1939). By its very nature of being composed of keratin, the intertubular horn is likely to hold at least 20% of the bound water within the hoof. This is the proportion of bound water Imokawa *et al.,* (1991) found in the keratin within human *stratum corneum.* Thomason *et al.,* (1992) suggested that intertubular horn contributed more to hoof strength, stiffness and fracture toughness than the tubules and this may be due in part to the bound water capacity of the intertubular horn.

It could be that the drier environmental conditions for the summer months accentuate the bound water gradient across the hoof wall, and the lower humidity environment to the outside of the hoof causes bound water to be lost to the external environment. This could involve both bound water and free water being lost, dependent on the relative humidity and temperature (Section 4.1.1). Bound water from the outer two zones of the hoof could be also lost once the hoof has lost any free water present and equilibrated to the new relative humidity conditions. The exact humidity and temperature at which the bound water contained within hoof wall material is lost to the environment requires further investigation and this point is discussed in more detail in Chapter 6.

In the current study fully hydrated hoof horn had to be analysed to establish the water holding capacity and fractionation of the hoof zones, as reproducible and measurable peaks could not be produced from fresh hoof horn. Further studies need to be carried out to establish at exactly what moisture content (%) the hoof horn exhibits free water. This would involve hydrating hoof horn in a series of relative humidity environments prior to running samples through the DSC machine to determine bound water content (mg/mg dry matter).

4.5 Conclusions

- The full hoof wall depth BWC (mg/mg dry matter) of donkeys, mules and ponies tends to be at its lowest in summer, with hoof BWC (mg/mg dry matter) in July and May being lower than in February and September.

- The zonal hoof wall bound water content (mg/mg dry matter) of donkeys, mules and ponies varies minimally between months for zone 1 and 2, however, zonal hoof wall BWC (mg/mg dry matter) for all species varies to a larger extent between months in zones 3 and 4.

- When zonal hoof wall BWC (mg/mg dry matter) was compared, it increased in an anterior-posterior direction towards the internal hoof structures, away from the outer hoof wall for all species. The rise in hoof BWC (mg/mg dry matter) from external zone 1 to internal zone 4 was on average 0.400 mg/mg for donkeys, 0.357 mg/mg for mules and 0.219 mg/mg for ponies.

- Ponies showed a characteristic anterior-posterior hoof bound water content (mg/mg dry matter) gradient with a gradual increase in BWC from zone 1 to zone 3 and then a larger increase between zones 3 and 4. The mule hoof BWC gradient was similar to ponies between zones 1 and 2 and then showed distinct divisions with larger increases between zones 2 and 3, and zones 3 and 4, which was more characteristic of the donkey hoof BWC gradient. This may indicate that the water binding characteristics of the mule hoof are some kind of hybrid between those of the pony and the donkey.

CHAPTER 5 – DONKEY, MULE AND PONY HOOF GROWTH

5.1 Introduction

As has already been mentioned in section 1.5.1, there has been a number of studies concerning hoof growth in horses and ponies, but there have been no published studies concerning the hoof growth rates of donkeys and mules. Also mentioned in section 1.5.1 was the distinction to be made between hoof growth and hoof growth rate. What is often of interest to the owner of the horse or donkey is the replacement time for, or the growth rate of, the hoof capsule. This is especially significant when growing out an injury or a hoof defect (Reilly *et al.*, 1998a). As Florence and MacDonnell (2006) only studied hoof wear in the summer months, further research incorporating the full calendar year is required to ascertain the full implications of climate and season on hoof wear rates.

5.1.1 Hoof growth and replacement time

Replacement time is usually measured in days or months and is used to give the owner an indication of when their animals will have grown the injury/defect out and have a completely new hoof capsule. It has been suggested by Josseck *et al.*, (1995) that there are differences in growth rates between midline, lateral sidewall and palmar/plantar sites in horse hooves. Unfortunately, they did not give any figures for individual sites in that study, only a mean of dorsal and lateral quarter measurements.

Following the work of Reilly *et al.*, (1998a), most authors have used the midline dead centre (MDC) sampling site, as it is clearly defined and gives precision of measurement, enabling accurate comparisons to be made. The literature search for this thesis revealed only one study which had measured hoof growth rates around the full circumference of the hoof. Frackowiak & Komosa (2006) described collateral part-two and movable part-two as well as dorsal measurement sites for their study into the dynamics of hoof

growth of Konik horses, although there was no diagram illustrating exactly where these locations were on the hoof, so it is unclear whether these measurement sites are equivalent to the toes, quarters and heels measurement sites. Through the establishment of a reproducible method for determining the quarter and heel measurement sites (See Figure 5.2.1), it is hoped that the growth rate for the full hoof circumference can be ascertained for donkeys, mules and ponies.

Josseck *et al.*, (1995) reported a decreased growth rate in winter for horse hoof horn and attributed this to reduced digital blood flow. Frackowiak & Komosa (2006) also found seasonal fluctuations in the hoof growth rate of Konik mares and stallions which decreased in winter. They attributed this reduced hoof growth rate to decreased metabolic function and changes in hormonal balance, associated with the drop in day length, temperature and the associated reduced activity of the horses.

Josseck *et al.*, (1995) also noted differences between individual horses, and between breeds, which would indicate that differences between the hoof horn growth of the three equid species may be likely.

5.1.2 Hoof wear rate

Also of importance, particularly to donkey and mule owners both abroad and in the UK, is the hoof wear rate. The vast majority of working donkeys and mules are unshod, and so a positive growth:wear ratio must be maintained so that the animals do not get footsore or go lame. Recently, an increasing number of practitioners, both approved farriers and lay equine podiatrists, are practising bare foot and hoof trimming rather than the traditional method of shoeing ridden horses. This is based on observations that horses which are self-maintaining in terms of hoof trimming have relatively few hoof problems (Jackson, 1997; Ovnicek, 2003).

Wear rate can be calculated from the measurement of full hoof wall height pre and post trimming (see Figure 5.2.2), and then calculating the growth minus wear at the next measurement date. Any difference in hoof wall height will be net growth, if positive or wear, if negative, for that period. Dividing the result by the number of days between measurements will give the rate of both in mm/day. This calculation takes into consideration the fact that the hoof wall has grown down as well as been worn away at the bearing border. A positive figure will indicate that growth has exceeded wear, and therefore there is net growth. A negative figure will indicate that wear has exceeded growth, and therefore there is net wear.

As reported previously in Section 1.5.6, one study reporting the hoof wear rate of semi-feral Shetland sized ponies in Pennsylvania, USA (Florence & McDonnell, 2006), was found in the literature review for this thesis. Unfortunately, they analysed growth and wear rates only for the months June – September, and not a full calendar year. However, hoof wear rates have been studied in detail in other species, particularly cattle. Hahn *et al.,* (1986) noted that it becomes more important to monitor wear rates in cattle when they are confined on abrasive surface such as concrete, as new tissue and horn must be grown at an increased rate to replace the horn worn away. Failure of adequate hoof horn replacement could result in lameness or reduced performance. Hahn *et al.,* (1986) found wear rates in cattle of 0.19mm/day and Clarke & Rakes (1982) found cattle hoof wear varied with season from 0.23mm/day summer down to 0.15mm/day in winter. Florence & McDonnell (2006) found that, for ponies, the daily wear rate varied from 0.52mm/day in July to 0.24mm/day in September. The July wear rate is considerably higher than those found in cattle, although the September pony hoof wear rate is similar to that found by Clarke & Rakes (1982) for cattle during the summer months. As Florence and MacDonnell (2006) only studied hoof wear in the summer months, further research incorporating the full calendar year needs to be carried out to ascertain the full implications of climate and season on equine hoof wear rates.

5.1.3 Aims

The aim of this part of the study was to:

- establish a reproducible method for determining the quarter and heel measurement sites

- establish the baseline growth rate for donkey, mule and pony hoof horn on the left fore hoof over the period of a calendar year

- establish if there is a seasonal influence affecting donkey, mule and pony hoof horn growth rates

- establish whether there are significant differences in hoof growth rate around the circumference of the hoof for donkeys, mules and ponies

- establish the baseline wear rate for donkey, mule and pony hoof horn on the left fore hoof over the period of a calendar year

- establish if there is a seasonal influence affecting donkey, mule and pony hoof horn wear rates

- establish whether there are significant differences in hoof wear rate around the circumference of the hoof for donkeys, mules and ponies

- establish the normal baseline net hoof growth/wear rate of the donkey, mule and pony hoof horn on the left fore hoof over the period of a calendar year

- establish if there is a seasonal influence affecting donkey, mule and pony net hoof horn growth/wear rate

- establish whether there are significant differences in net hoof growth/wear rate around the circumference of the hoof for donkeys, mules and ponies

- establish how long it takes to completely replace the hoof for donkeys, mules and ponies maintained in a temperate environment.

5.2 Method

5.2.1 Experimental design

The experimental design was a factorial (2x3x6), three-way mixed design, as described on page 61.

Factor 1 = Farm (Brookfield, Town Barton)

Factor 2 = Species (donkey, mule and pony)

Factor 3 = Sampling Time (February, April, May, July, September, November)

Hoof growth was measured over a 12 month period, from 18 December 2002 until 23 November 2003, in a trial using donkeys, mules and ponies, kept in mixed groups and maintained in a temperate climate at the Donkey Sanctuary farms, at Brookfield and Town Barton in the UK. Measurements were made every 10 weeks from the left fore hoof, and the hoof growth, wear and renewal rates were calculated for all the sampling sites.

As mentioned in Section 2.2, animals under the age of 4 years and over the age of 35 were excluded from the trial. This was to ensure that the trial group only contained adult, non-veteran animals, to reduce the number of variables which may affect the hoof growth/wear rate. Veterinary and movement details were checked to confirm that the animals had been at the Donkey Sanctuary for the previous 6 months to establish baseline conditions, and that they were not suffering from any long term illnesses. All hooves had been previously subject to regular trimming and were checked for the absence of large cracks or any hoof deformation. All animals were treated as they normally would be by the Sanctuary with regard to feeding, worming and hoof trimming.

As the study lasted for one year, the hoof growth measurement spanned the periods of indoor and outdoor activity of the animals. During the winter months (November – April) the animals were housed in large indoor barns on deep litter straw bedding, with

concrete run out areas, and fed *ad lib* haylage. During the summer months (May-October) the animals were turned out into free range grass paddocks.

5.2.2 Definition of measurement sites

Measurements were made on the left fore hoof only. Measurement sites were located at the midline dead centre (MDC), medial and lateral quarters and medial and lateral heels (see Figures 5.2.1 and 5.2.2), these were defined for all species. The MDC is defined as the line bisecting the foot, and is given by extending a line that bisects the frog onto the dorsal hoof wall (Reilly *et al.,* 1998a).

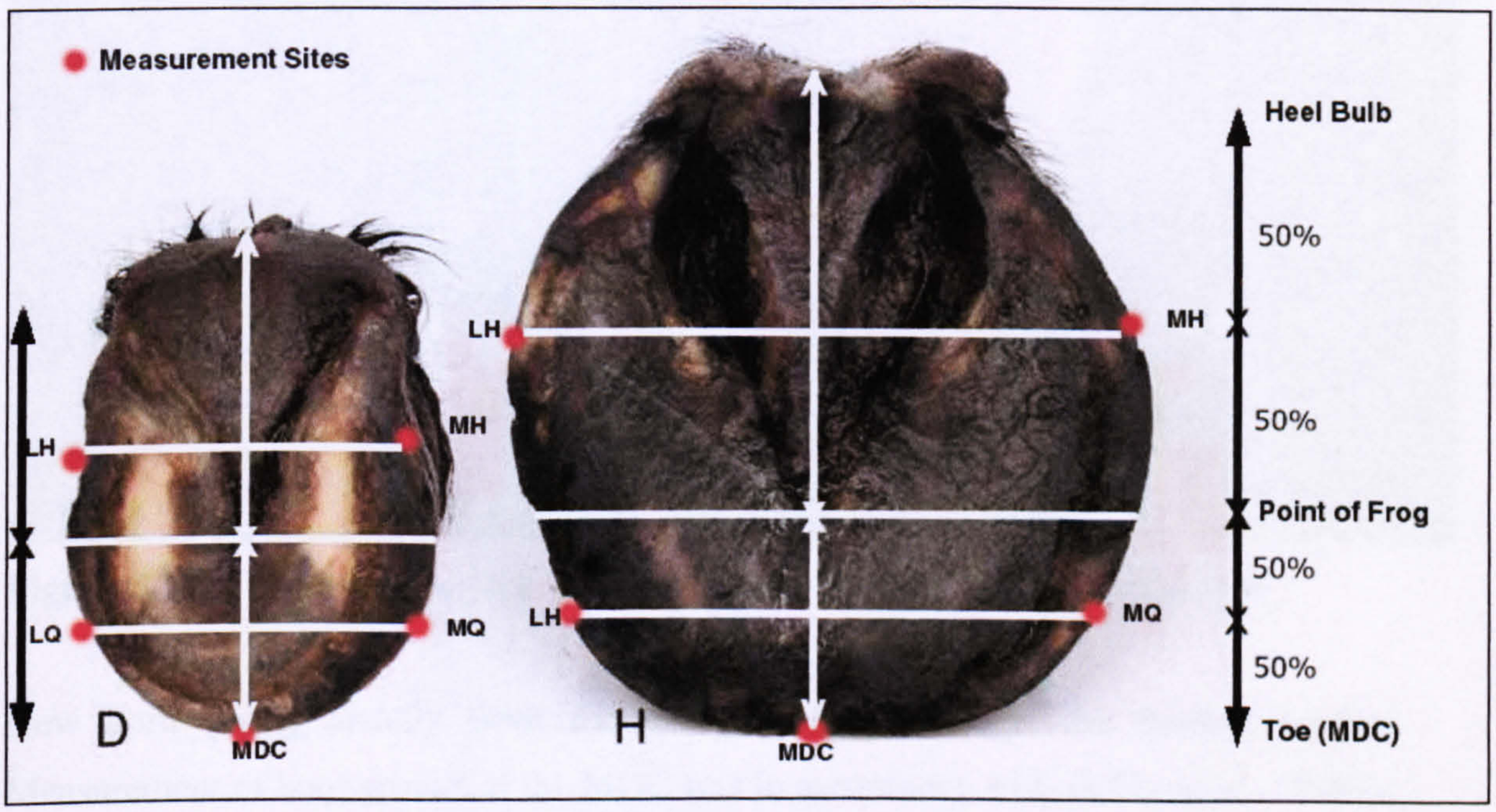


Figure 5.2.1 Definition of hoof capsule measurement sites for growth measurement ventral view. Donkey (D) hoof on the left, horse (H) hoof on the right. Lateral heel (LH), lateral quarter (LQ), midline dead centre (MDC), medial quarter (MQ) and medial heel (MH)

The heel measurements were made at sites 50% of the distance between the point of the frog and the bulb of the heel on the medial and lateral hoof walls. The quarter measurements were made at sites which were 50% of the distance between the point of

the frog and the toe (MDC) on the medial and lateral hoof walls. This made five sampling sites for each hoof (Figure 5.2.2).

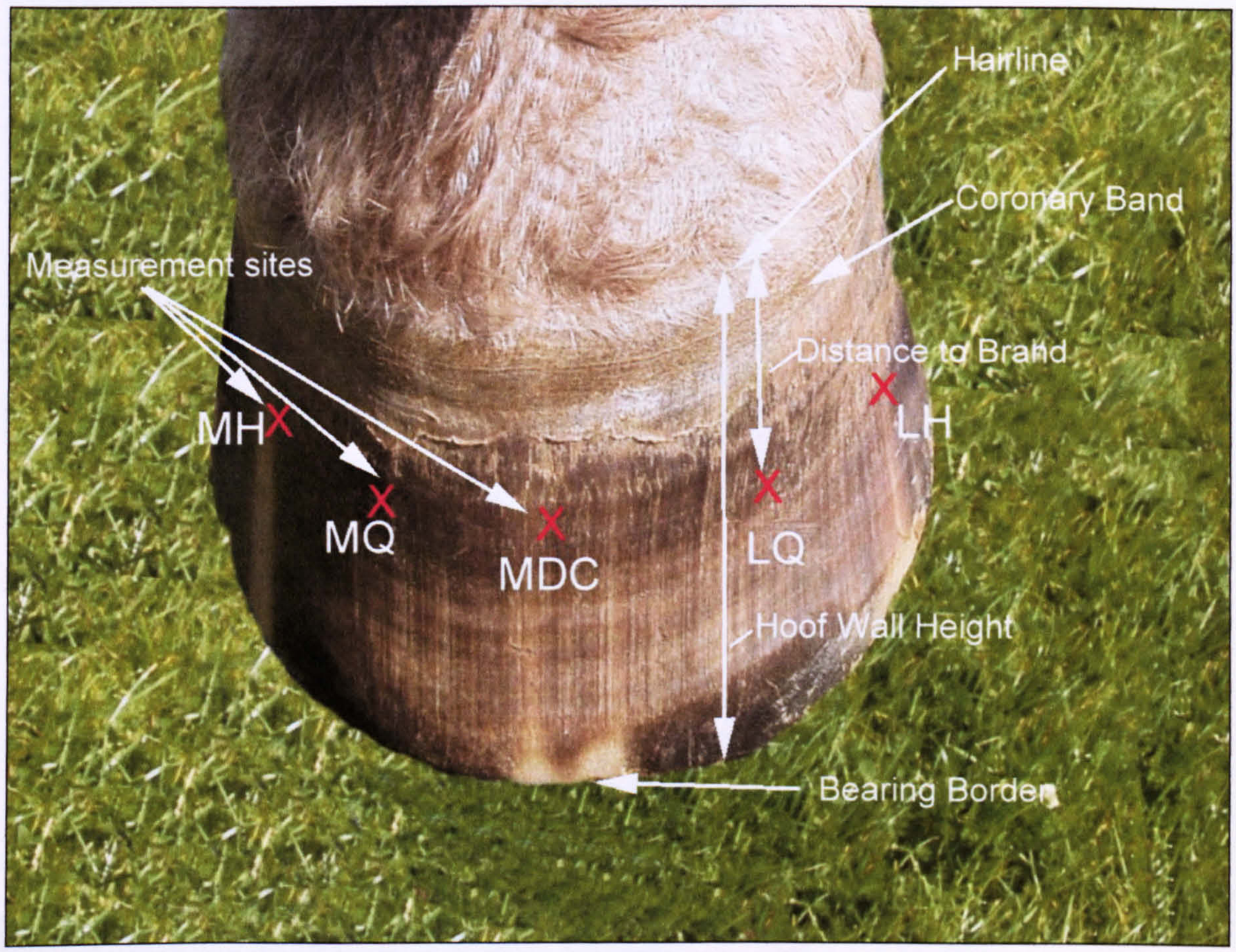


Figure 5.2.2 Hoof showing 5 hoof growth measurement sites – dorsal view

New horn grows distally from the coronary band towards the bearing surface. Measurement of hoof growth at the MDC was in accordance with Reilly *et al.,* (1998a). Measurements were also made at lateral and medial quarters and lateral and medial heels (see Figure 5.2.2) using the same method.

The hoof was marked with a marker pen at a given site, prior to superficial branding by the farrier (Figure 5.2.3) with a hot Philips screwdriver point, leaving a small 'X' mark in the outer hoof wall. The initial mark was made at a variable proximodistal point, between 1.5-2.5cm, from the hairline. The minimum distance from the hairline was 1.5cm in order to avoid damage to the periople or to horn production sites near the coronary band.

Figure 5.2.3 Farrier branding the left fore hoof

Digital vernier callipers (Mitutoyo (UK) Ltd) were then used to measure the distance from the hairline above the coronary band to the branded 'X' (distance to brand, see Figure 5.2.2), and also the hoof wall height (HWH) distance from the hairline to the bearing border of the hoof (see Figure 5.2.2). To ensure reproducibility of the measurements, the hair was turned back and measurements made from the roots of the hair (see Figure 5.2.4).

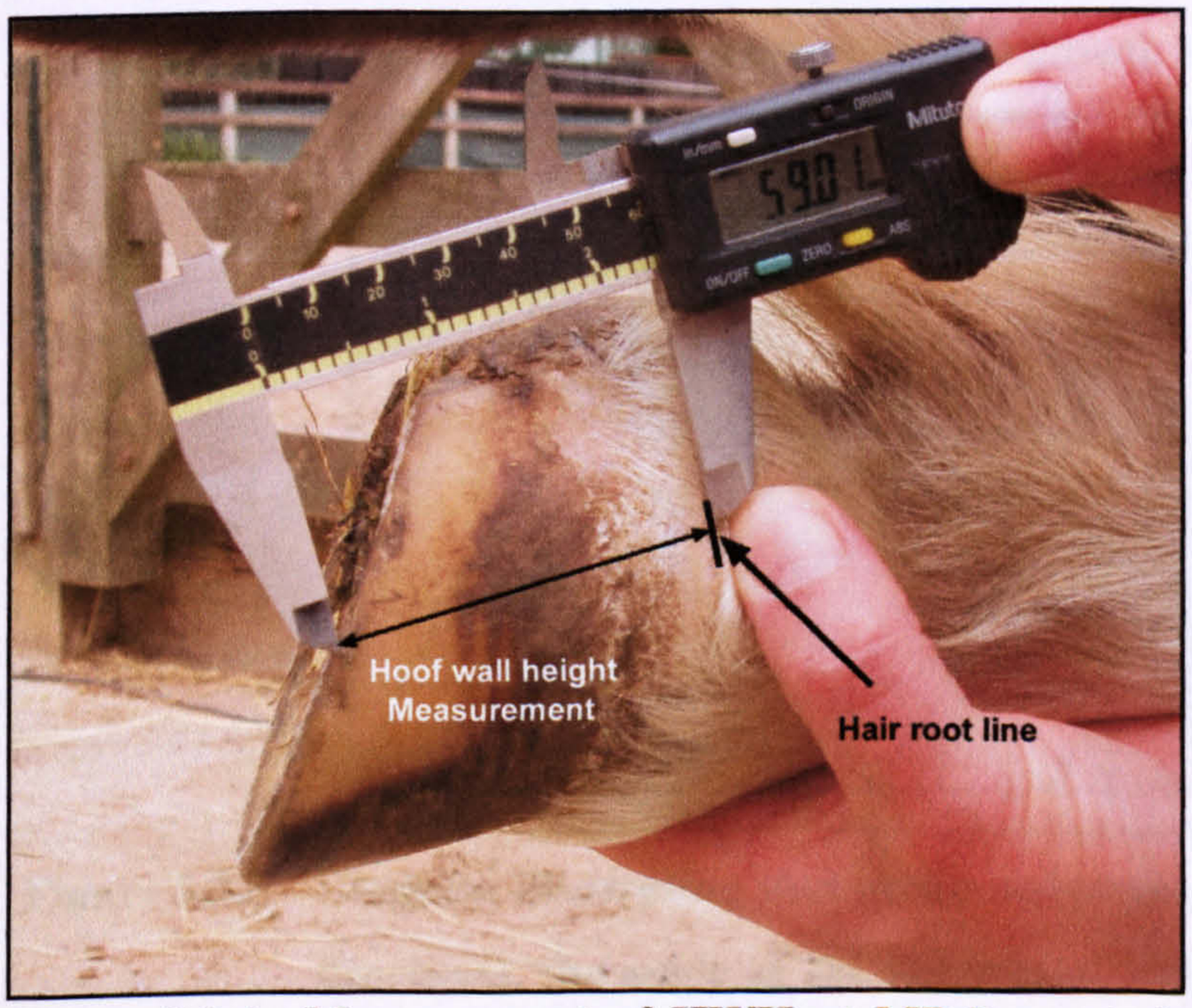


Figure 5.2.4 Measurement of HWH at MDC - note hair length turned back to expose the hair root line

Upon subsequent measurement occasions, these distances were re-measured prior to any routine hoof trimming. All measurements were made in line with the angle of tubule growth, to ensure continuity and reproducibility of measurements. The growth between sampling occasions was then calculated by subtracting the initial measurement from the subsequent measurement. Subtraction of the initial brand measurement from the subsequent measurement gave the total hoof growth for the period between farriery visits (usually 70 days). This was then divided by the number of days to give the growth rate for the period in mm/day.

Where re-branding was required because the 'X' marks were close to being grown distally out of the hoof, the measurements of growth were made as above, then the sites that needed re-branding were marked using marker pen. Care was taken to ensure that the marks were in line with the tubule angle, directly above the previous brands. Re-branding and re-measurement of the distance to the brand and hoof wall height was then carried out as above. These newly branded measurement sites provided the baseline for subsequent measurements. The angle of the tubule growth can be seen as fine longitudinal graining in the hoof wall (Figure 5.2.2) and as the angle of the hoof wall decreases from the MDC at the toe, through the quarters to the heels, so does the anteroposterior angle of the hoof wall tubules (Figure 5.2.5). Measurement in line with the tubule angle ensured accurate and reproducible growth measurement.

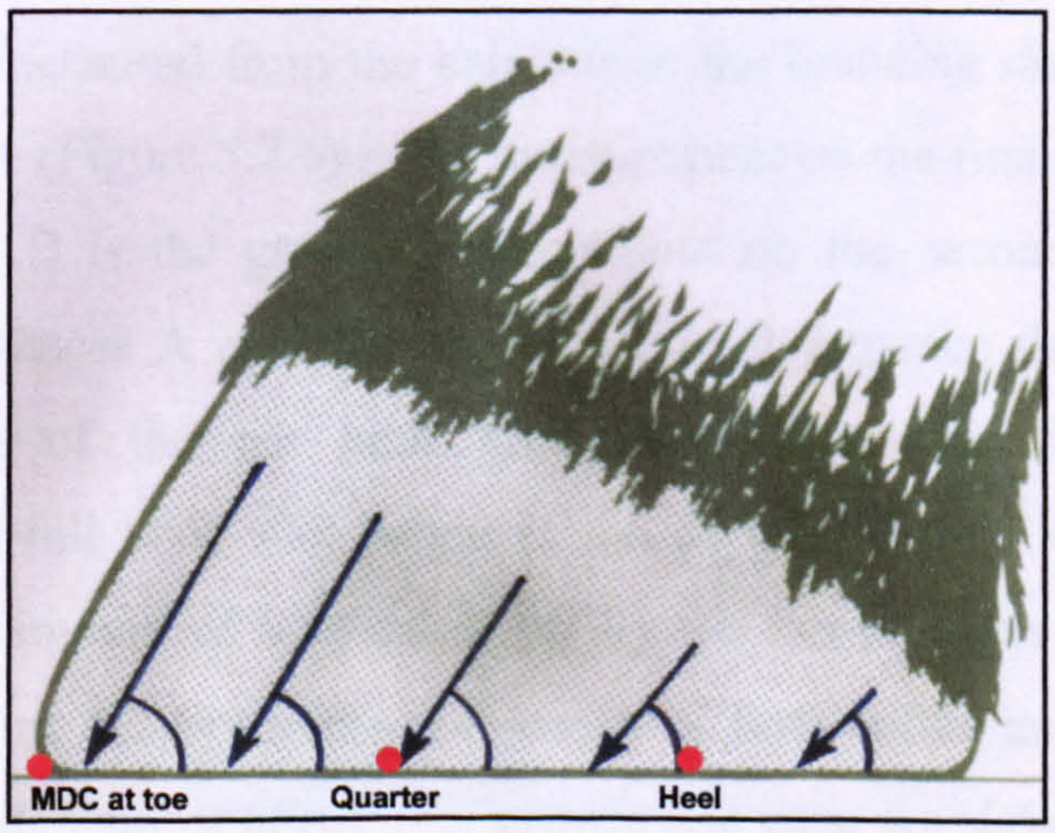


Figure 5.2.5 Diagram illustrating that as the anteroposterior angle of the tubules decreases, the angle of the outer hoof wall decreases (after Jackson, 1997)

5.2.3 Calculation of wear and growth rates

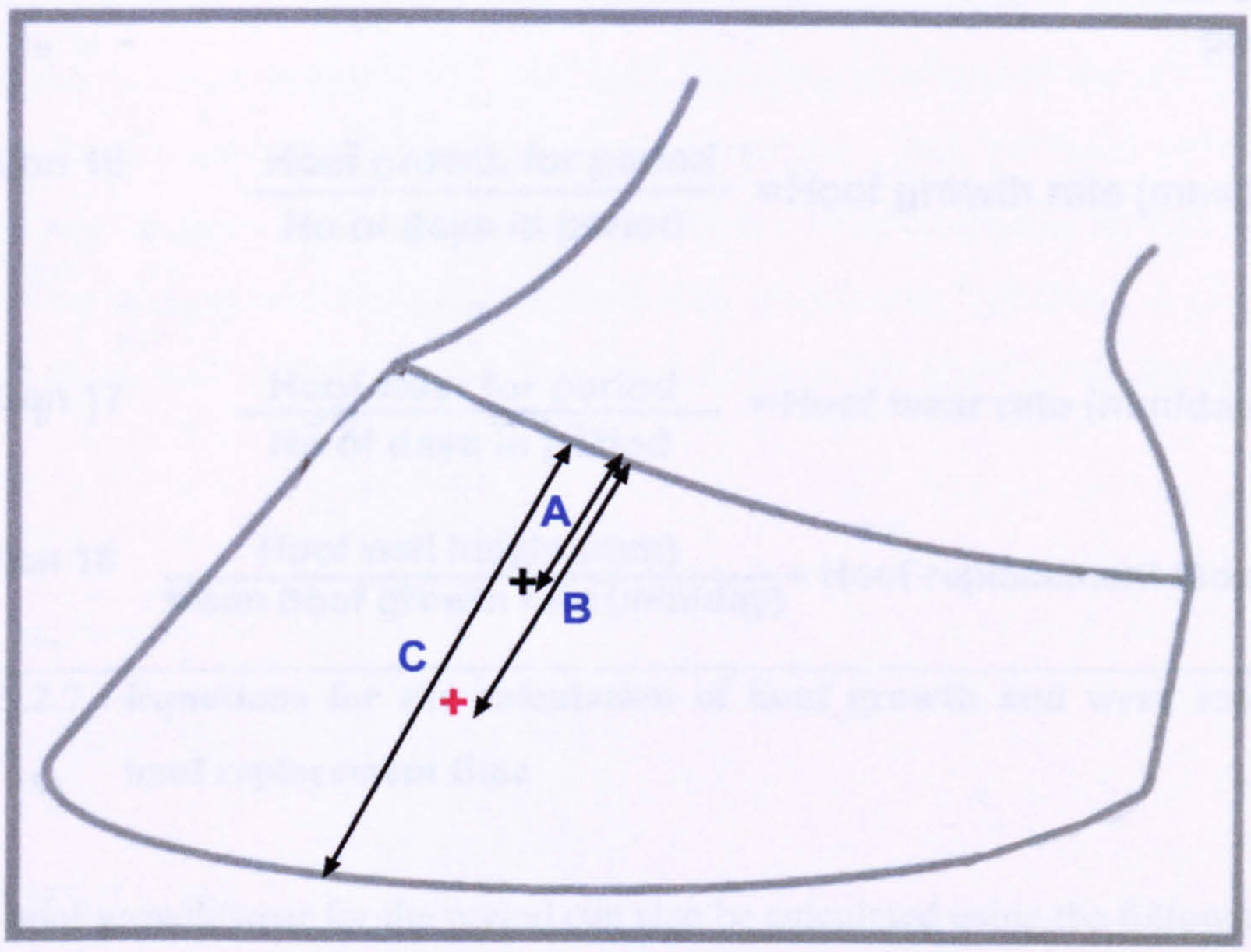


Figure 5.2.6 Diagram illustrating that as the measurement of full hoof wall height (C), growth 1st date (A) and growth 2nd date (B)

Growth can be measured from the hair line to the branding marks. The distance A to the brand mark + (Figure 5.2.6) is the measurement on the first date, and the distance to the brand mark B is the growth measurement on the second date. The difference between the distances A and B is the total growth between the first and second date. The calculation of the net hoof growth/wear rate (mm/day) also involves the measurement of full hoof wall height (C on Figure 5.2.6). Care has to be taken to account for the amount of hoof taken off by the farrier when trimming, so hoof wall height (C in Figure 5.2.6) needs to be measured both before and after trimming. For a set period, the calculations of net hoof growth and wear are shown in Figure 5.2.7.

Equation 14	**B - A = Hoof growth for the current period**	
Equation 15	**Post-trim C (previous) - Pre-trim C (current) = Hoof wear for current period**	
Equation 16	$\frac{\text{Hoof growth for period}}{\text{No of days in period}}$	**= Hoof growth rate (mm/day)**
Equation 17	$\frac{\text{Hoof wear for period}}{\text{No of days in period}}$	**= Hoof wear rate (mm/day)**
Equation 18	$\frac{\text{Hoof wall height (mm)}}{\text{Mean hoof growth rate (mm/day)}}$	**= Hoof replacement time (days)**

Figure 5.2.7 Equations for the calculation of hoof growth and wear rates, and hoof replacement time

The net hoof growth/wear for the period can also be calculated using the following:

Hoof growth rate – hoof wear rate = Net hoof growth/wear (mm/day)

Net growth/wear rates are very important, as if there is insufficient growth over an extended period of time, there may be injury to the structures of the foot and debilitating lameness may develop.

5.2.4 Statistical analysis

All data were analysed using SPSS 14 for Windows (SPSS Inc., Chicago, Illinois 60606). The data sets were checked for normality using normal probability plots. Data was evaluated for reduction using Factor Analysis and then General Linear Models were used to analyse the reduced data set. Significance for all analyses was assessed at a minimum of $p < 0.05$.

5.3 Results

There were 6 animals of each species on each farm. The hoof growth for these animals was measured on six occasions, at 5 hoof wall sampling locations of the left fore hoof. Hoof growth rate (mm/day), hoof wear rate (mm/day) and net hoof growth/wear (mm/day) (see Sections 5.3.2-12) were then calculated for each species at each farm, for each measurement period. Hoof replacement time (days) (see Sections 5.3.13-15) was calculated at the end of the trial period using a mean of the growth rate for the whole year.

5.3.1 Hoof growth rate analysis results

Mixed between-within subjects analyses of variance were conducted. There were no significant differences ($p > 0.05$) in hoof growth rate (mm/day) between hoof wall locations, farms, gender or hoof pigment. There were, however, significant differences ($p < 0.05$) in hoof growth rate (mm/day) between species and between months for donkey hoof.

There was no overall trend in hoof growth rate (mm/day) for any species. However, the mule and pony hoof growth rate (mm/day) appeared to follow a distinctive pattern where hoof growth rate (mm/day) fluctuated with peaks in February, May and September, and troughs in April, July and November (Figure 5.3.3). The hoof growth rate (mm/day) of donkey hoof was lower than that of both pony and mule hoof for all months, and the difference was significant ($p < 0.05$) in September and November.

Due to the large size of the hoof growth data set, factor analysis was performed on the raw data. The full set of hoof growth (mm/day) results is to be found in Appendix 3.

The hoof growth data is presented in the following order, to present the data logically, and to extract all different comparisons:

5.3.2 Factor analysis of hoof growth rate (mm/day) raw data set

5.3.3 General linear model analysis of the effects of species, farm, month, gender and pigment on hoof growth rate (mm/day)

5.3.4 General linear model analysis of hoof growth rate (mm/day) between months

5.3.2 Factor analysis of the hoof growth rate (mm/day) raw data set

Factor analysis was conducted in order to reduce the data set. Analyses were carried out on the whole of the data for each month. The 5 items of the hoof growth rate analysis for February were subjected to principal components analysis (PCA) using SPSS Version 15. Principal components analysis revealed two components with an eigenvalues exceeding 1, explaining 68% of the variance. An inspection of the scree plot revealed an unclear break after the first component (Figure 5.3.1).

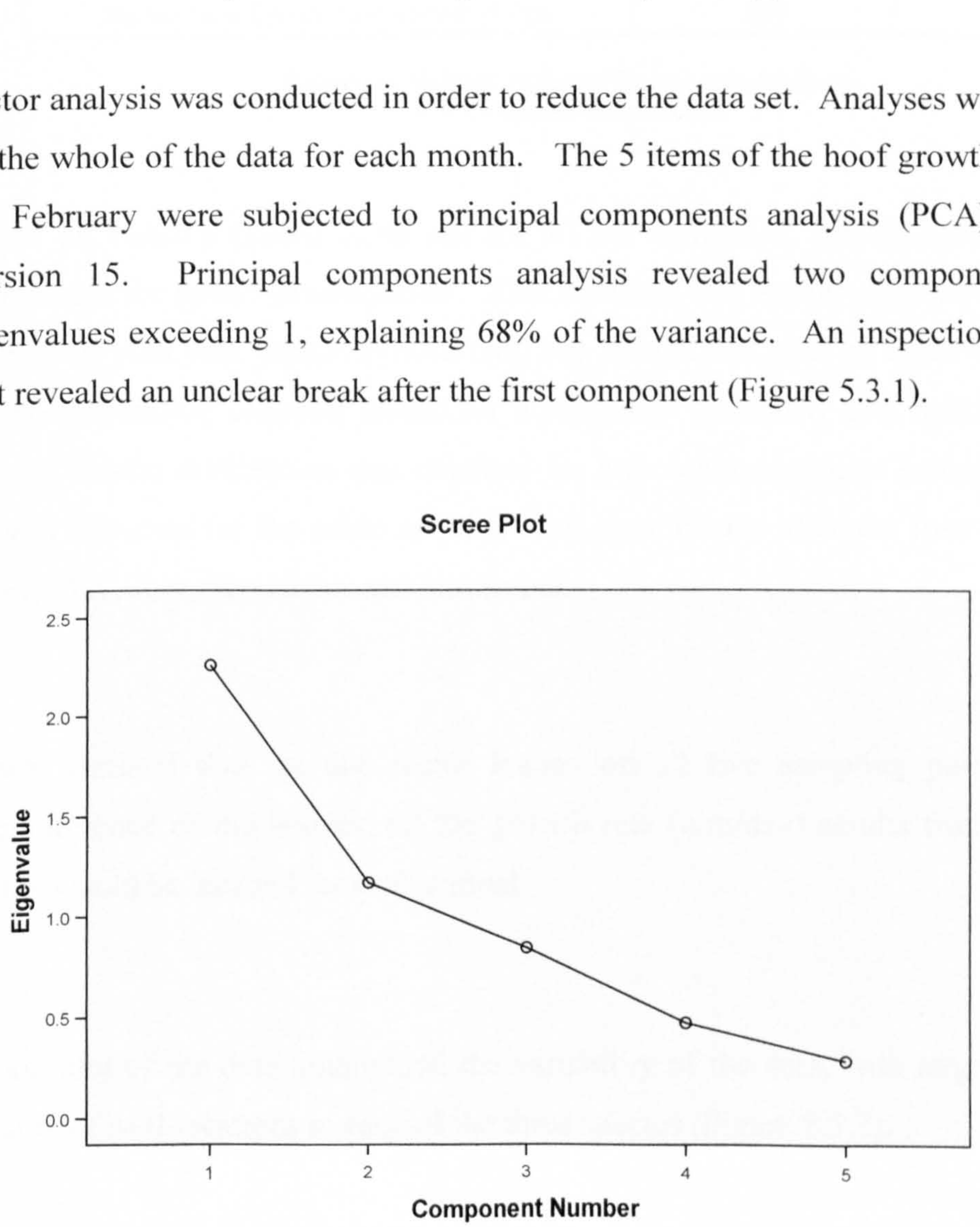


Figure 5.3.1 Scree test plot of February hoof growth rates (mm/day)

Most of the loading was on the first component (Table 5.3.1).

Table 5.3.1 Component matrix of hoof growth rate (mm/day) of hoof wall sample sites for February

Component Matrix (a)

	Component	
	1	2
Lateral Heel Growth rate (mm/day) Feb	**.777**	.119
Lateral Quarter Growth rate (mm/day) Feb	**.808**	-.417
Midline Dead Centre Growth rate (mm/day) Feb	**.797**	-.255
Medial Quarter Growth rate (mm/day) Feb	.298	**.891**
Medial Heel Growth rate (mm/day) Feb	**.528**	.344

Extraction Method: Principal Component Analysis.
a 2 components extracted.

Based on Catell's (1966) scree test for all the months, it was decided to retain one component for further investigation. This decision was further supported by the results of Parallel Analysis, which showed only one component with an eigenvalue exceeding the corresponding criterion values for a randomly generated data matrix of the same size. A similar distribution was obtained for principal component analysis on the hoof growth rate data for the other months, with clear breaks after the first component for April, May, July, September and November.

It was decided that, as one factor loaded on all five sampling points around the circumference of the hooves, all the growth rate (mm/day) results from the sampling points would be meaned for each animal.

A box plot of the data highlighted the variability of the data, with large error bars for most hoof wall locations in each of the three species (Figure 5.3.2).

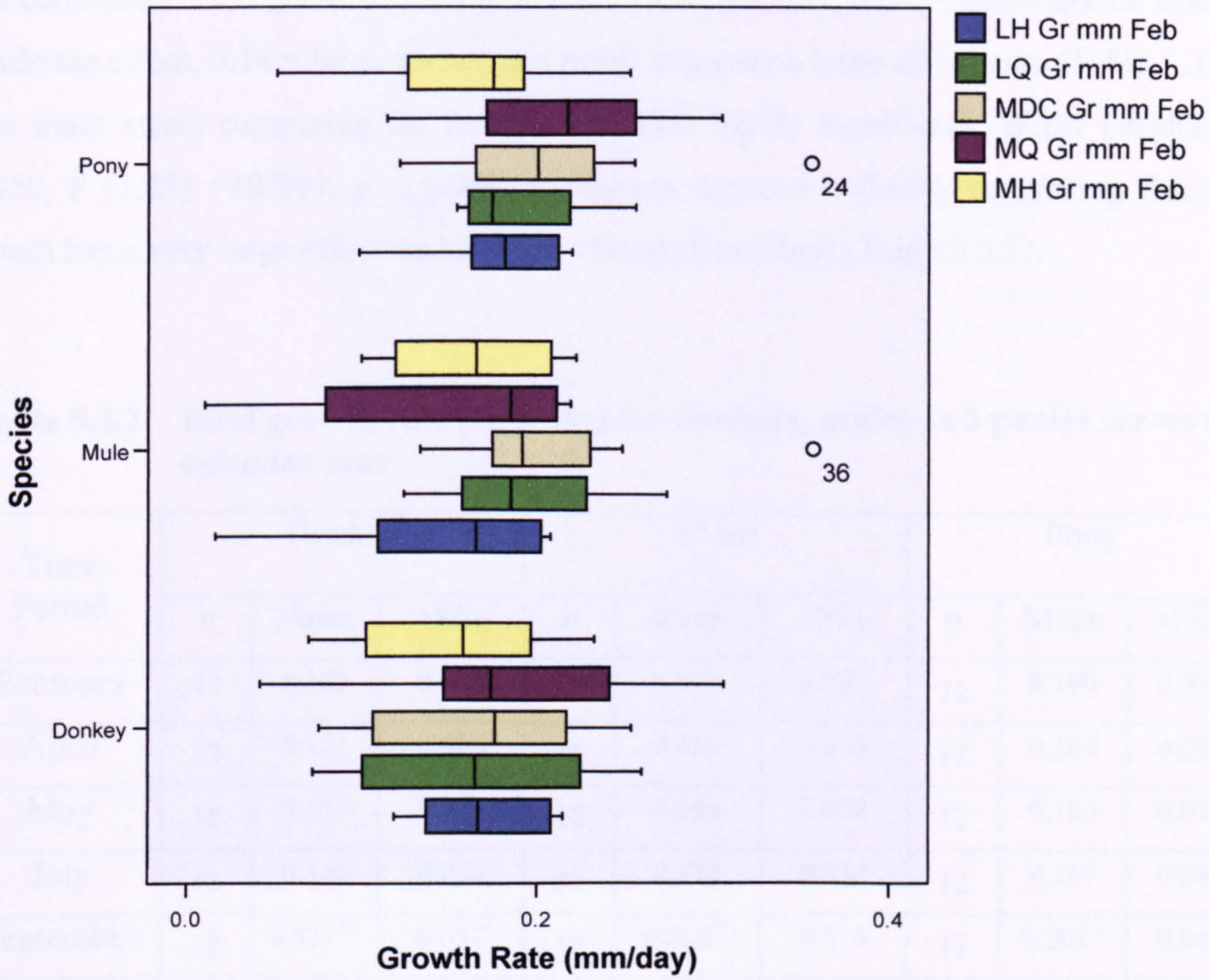


Figure 5.3.2 Boxplot of hoof growth rate (mm/day) of hoof wall circumferential samples for February for donkeys, mules and ponies

5.3.3 General linear model analysis of the effects of species, farm, month, gender and pigment on hoof growth rate (mm/day)

Mixed between-within subjects analyses of variance were conducted on the hoof growth rate (mm/day) results, to assess the impact of farm, gender and species on hoof growth rate (mm/day), across the calendar year (February – November sampling occasions). The mixed between-within subjects analyses of variance for hoof growth rate (mm/day) showed no significant interaction between the month and hoof pigmentation or farm location. There was, however, a significant interaction between month and species, Wilks Lambda = 0.400, $F(10,58) = 3.368$, $p < 0.005$, partial eta squared = 0.367. Using

the commonly used guidelines proposed by Cohen (1988): 0.01 = small effect, 0.06 = moderate effect, 0.14 = large effect, this result suggests a large effect size (Table 5.3.2). The main effect comparing the months was also highly significant, Wilks Lambda = 0.350, F (5,29) =10.749, $p < 0.001$, partial eta squared = 0.650, suggesting that the month has a very large effect on hoof growth rate (mm/day) (Table 5.3.2).

Table 5.3.2 Hoof growth rate (mm/day) for donkeys, mules and ponies across the calendar year

Time Period	Donkey			Mule			Pony		
	n	Mean	$\pm SE_D$	n	Mean	$\pm SE_D$	n	Mean	$\pm SE_D$
February	12	0.162	0.045	12	0.171	0.037	12	0.190	0.029
April	12	0.121	0.028	12	0.160	0.053	12	0.164	0.058
May	12	0.153	0.045	12	0.196	0.058	12	0.180	0.078
July	12	0.146	0.024	12	0.174	0.034	12	0.167	0.040
September	12	0.135 [bc]	0.037	12	0.205 [b]	0.036	12	0.201 [c]	0.045
November	12	0.135 [bc]	0.031	12	0.158 [b]	0.029	12	0.162 [c]	0.034

Hoof growth rate of species, within rows, means with same superscripts differ (b,c = $p < 0.005$) between species

5.3.4 General linear model analysis of hoof growth rate (mm/day) between months

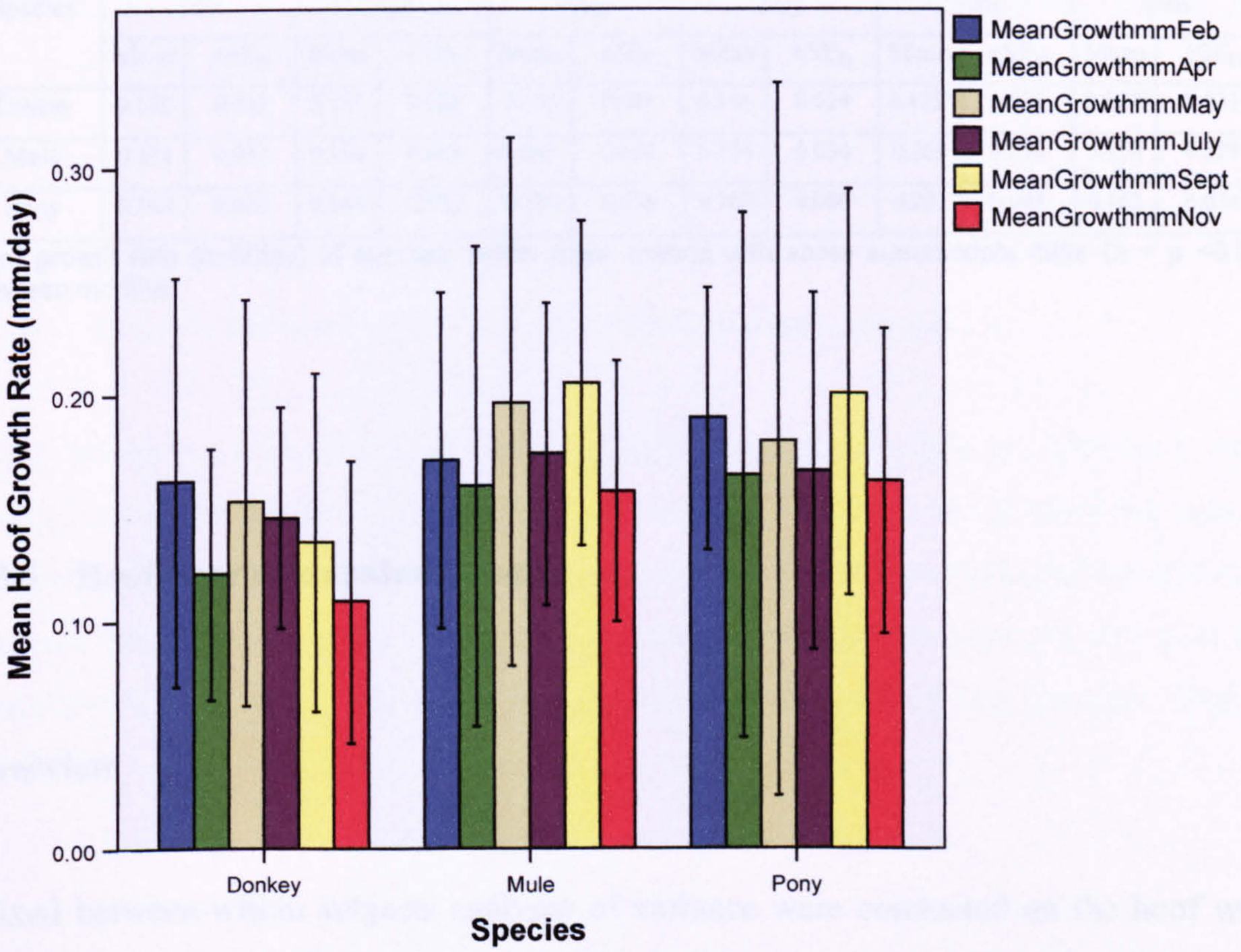


Figure 5.3.3 Hoof growth rate (mm/day) for donkeys, mules and ponies across the calendar year (n = 12 for all species/months)

There were no significant differences ($p > 0.05$) in hoof growth rates (mm/day) between months for mule and pony hoof growth, although both species follow a distinctive pattern where hoof growth rate fluctuates with peaks in February, May and September, and troughs in April, July and November (Figure 5.3.3). Donkeys do not appear to follow the same pattern of hoof growth rate (mm/day) fluctuation; the hoof growth rate declines from May to November, with a significant drop ($p < 0.05$) in hoof growth rate (mm/day) from 0.135mm/day ±0.037 SE_D in September to 0.107mm/day ±0.031 SE_D in November ($p < 0.05$) (Table 5.3.3).

Table 5.3.3 Hoof growth rate (mm/day) for donkeys, mules and ponies across the calendar year (n = 12 for all species/months)

Species	Feb		Apr		May		July		Sept		Nov	
	Mean	$\pm SE_D$	Mean	$\pm SE_D$	Mean	$\pm SE_D$	Mean	$\pm SE_D$	Mean	$\pm SE_D$	Mean	$\pm SE_D$
Donkey	0.162	0.045	0.121	0.028	0.153	0.045	0.146	0.024	0.135[a]	0.037	0.107[a]	0.031
Mule	0.171	0.037	0.160	0.053	0.196	0.058	0.174	0.034	0.205	0.036	0.158	0.029
Pony	0.190	0.029	0.164	0.058	0.180	0.078	0.167	0.040	0.201	0.045	0.162	0.034

Hoof growth rate (mm/day) of species, within rows, means with same superscripts differ (a = $p < 0.05$) between months

5.3.5 Hoof wear rate analysis results

Overview

Mixed between-within subjects analyses of variance were conducted on the hoof wear rate (mm/day) data. There were no significant differences ($p > 0.05$) in hoof wear rate (mm/day) between months, farms, gender or hoof pigment. There were, however, significant differences ($p < 0.05$) in hoof wear rate (mm/day) between hoof wall locations for all three species.

There was no overall trend in hoof wear rate for all species, however the donkey hoof wear rate appeared to follow a similar distinctive pattern to the pony and mule hoof growth rate. The donkey hoof wear rate fluctuated with peaks in April, July and November and troughs in February, May and September.

Due to the large size of the hoof wear data set, factor analysis was performed on the raw data. The full set of hoof wear results can be found in Appendix 4. The data are presented in the following order, to present the data logically and to extract all different comparisons:

5.3.6 Factor analysis of hoof wear rate (mm/day) raw data set

5.3.7 General linear model analysis of hoof wear rate (mm/day) between hoof wall locations

5.3.8 General linear model analysis of the effects of species, farm, month, gender and pigment on hoof wear rate (mm/day) at each hoof wall location

5.3.6 Factor analysis of the hoof wear rate (mm/day) raw data set

Factor analysis was conducted in order to attempt to reduce the data set. Analyses were carried out on the whole of the data for each month. The 30 items (6 sampling dates x 5 locations) of the hoof wear rate analysis were subjected to principal components analysis (PCA) using SPSS Version 15. Principal components analysis revealed ten components with eigenvalues exceeding 1, explaining 80% of the variance (Figure 5.3.4).

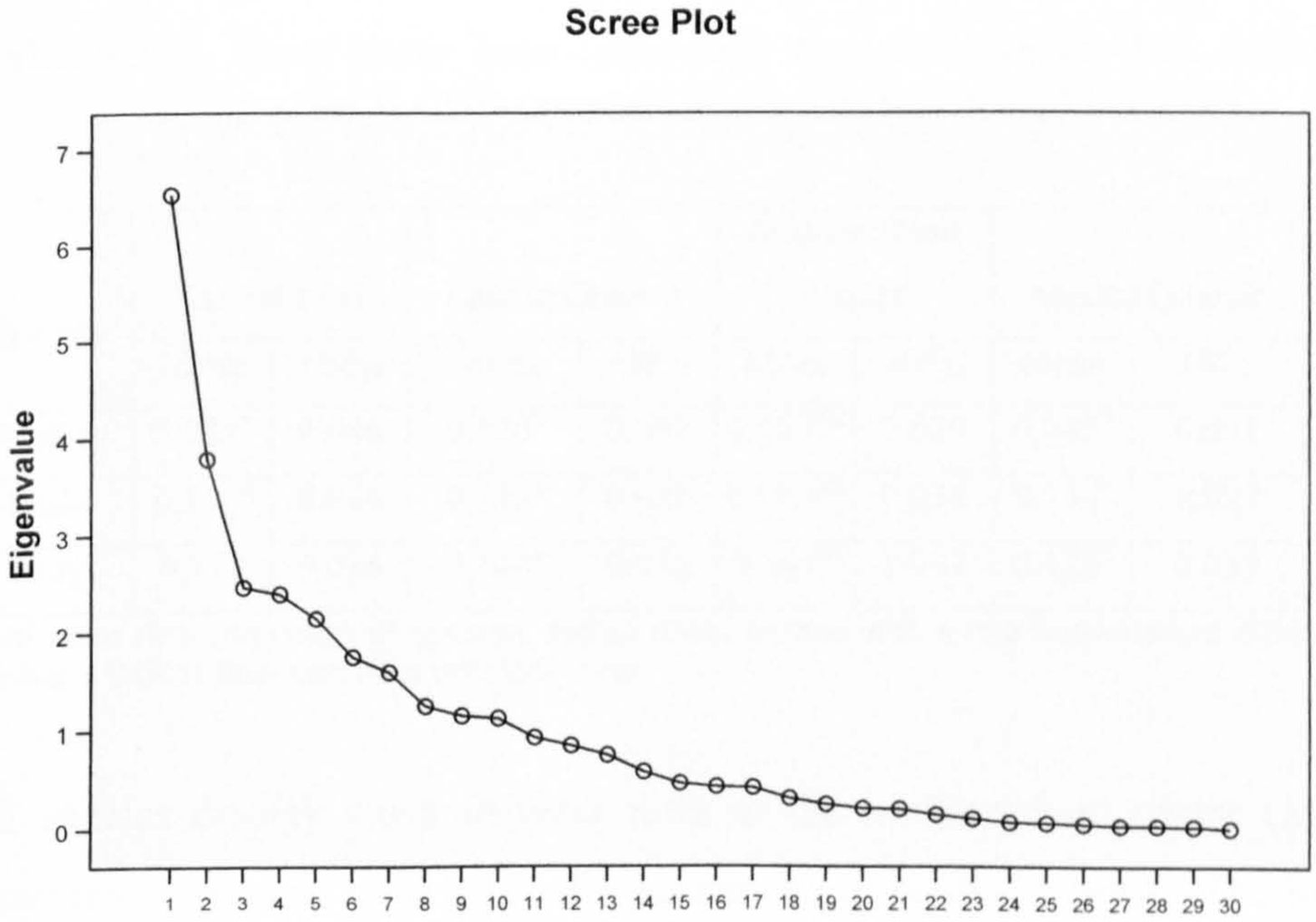


Figure 5.3.4 Scree test plot of hoof wear rate (mm/day) all measurements

Because the factor analysis results showed unclear loading, it was decided to retain all the hoof wear rate (mm/day) raw data for further investigation using general linear mixed between-within subjects analyses of variance.

5.3.7 General linear model analysis of hoof wear rate (mm/day) between hoof wall locations

Mixed between-within subjects analyses of variance were conducted on the hoof wear rate (mm/day) results to assess the impact hoof wall location on hoof wear rate (mm/day). The mixed between-within subjects analyses of variance for hoof wear rate (mm/day) showed no significant interaction between the hoof wall location and species. There was, however, a significant main effect comparing the hoof wall locations, Wilks Lambda = 0.621, $F(5,29) = 3.536$, $p < 0.05$, partial eta squared = 0.293, suggesting that the hoof wall location has a very large effect on hoof growth rate (mm/day) (Table 5.3.4).

Table 5.3.4 Hoof wear rate (mm/day) for donkeys (n=12), mules (n=11) and ponies (n=10) around the circumference of the hoof

Species	Lateral Heel		Lateral Quarter		Midline Dead Centre		Medial Quarter		Medial Heel	
	Mean	$\pm SE_D$	Mean	$\pm SE_D$	Mean	$\pm SE_D$	Mean	$\pm SE_D$	Mean	$\pm SE_D$
Donkey	0.084^{a}	0.048	0.120^{a}	0.038	0.150^{abc}	0.039	0.085^{b}	0.051	0.090^{c}	0.049
Mule	0.111^{d}	0.046	0.156^{a}	0.028	0.195^{bde}	0.034	0.136^{b}	0.027	0.097^{ac}	0.051
Pony	0.113	0.036	0.144^{a}	0.032	0.165^{bd}	0.037	0.120^{b}	0.033	0.094^{ad}	0.041

Hoof wear rate (mm/day) of species, within rows, means with same superscripts differ (a,b,c = $p < 0.05$, d,e = $p < 0.001$) between hoof wall locations

All species display elevated wear rates at the midline dead centre (MDC) sampling locations, with wear rates gradually decreasing towards the heels. The MDC hoof wear rate was significantly higher ($p < 0.05$) than the hoof wear rate at the quarters and heels in all three species. Mule hoof had the highest hoof wear rate at the MDC (0.195

mm/day ± 0.034 SE_D) and donkey the lowest hoof wear rate at the lateral heel (0.084 mm/day ± 0.048 SE_D).

5.3.8 General linear model analysis of the effects of species, farm, month, gender and pigment on hoof wear rates (mm/day) at each hoof wall location

Due to significant differences between wear rates at the MDC, quarters and heels, mixed between-within subjects analyses of variance were conducted on the hoof wear rate (mm/day) results for all five hoof wall locations. These were performed to analyse the impact of month, farm, species, gender and pigmentation on hoof wear rate (mm/day) at each location for a full calendar year.

Table 5.3.5 General linear model analysis of hoof wear rate (mm/day) results and significance levels (donkey n=12, mule n =11, pony n=10)

Hoof wall location	Month	Species	Farm	Gender	Pigmentation
Lateral heel	ns	ns	ns	ns	ns
Lateral quarter	ns	ns	ns	ns	*
Midline dead centre	*	ns	***	ns	ns
Medial quarter	ns	ns	ns	ns	ns
Medial heel	**	***	*	*	ns

ns = not significant p > 0.05, * significant (p < 0.05), ** significant (p < 0.005), *** significant (p < 0.001)

Results of the general linear model analysis carried out on all the hoof wall location hoof wear results were variable and did not show any strong trends, with sporadic significant interactions ($p < 0.05$) at various hoof wall locations. The medial heel wear rate (mm/day) appeared statistically to be significantly affected by month, species, farm and gender. It is not known why this effect was demonstrated in the medial heel and not the other hoof wall locations. The results for the significance of pigmentation need to be treated with caution due to the ratio of pigmented (32) to non-pigmented (4)

hooves (Table 2.2.1). A repeat study with a larger sample size and more non-pigmented hooves would be required to draw any meaningful conclusions on the effect of pigmentation on hoof wear rate (mm/day).

5.3.9 Net hoof growth/wear rate analysis results

Overview

Mixed between-within subjects analyses of variance were conducted on the net hoof growth/wear rate (mm/day) data. There were significant interactions ($p < 0.05$) between the net hoof growth/wear rate (mm/day) and the sampling months and hoof wall locations. Subsequent mixed between-within subjects analyses of variance were performed on each hoof wall location, to analyse the impact of farm, species, gender and pigmentation on net hoof growth/wear rate (mm/day) for a full calendar year. There were no significant interactions ($p > 0.05$) between species, gender and pigmentation and the net hoof growth/wear rate (mm/day) for any of the sampling locations. However there were significant interactions between farm and net hoof growth/wear rate (mm/day) for midline dead centre ($p < 0.001$) and medial heel ($p < 0.05$). The data are presented in the following order, to present the data logically and to extract all different comparisons:

5.3.10 Factor analysis of the net hoof growth/wear rate (mm/day) raw data set

5.3.11 General linear model analysis of the effects of month and hoof wall location on net hoof growth/wear rates (mm/day)

5.3.12 General linear model analysis of the effects of species, farm, gender and pigment on net hoof growth/wear rates (mm/day)

5.3.10 Factor analysis of the net hoof growth/wear rate (mm/day) raw data set

Factor analysis was conducted in order to attempt to reduce the data set. Analyses were carried out on the whole of the data for each month. The 30 items (6 sampling dates x 5 locations) of the hoof wear rate analysis were subjected to principal components analysis (PCA) using SPSS Version 15. Principal components analysis revealed eleven components with eigenvalues exceeding 1, explaining 81% of the variance (Figure 5.3.4).

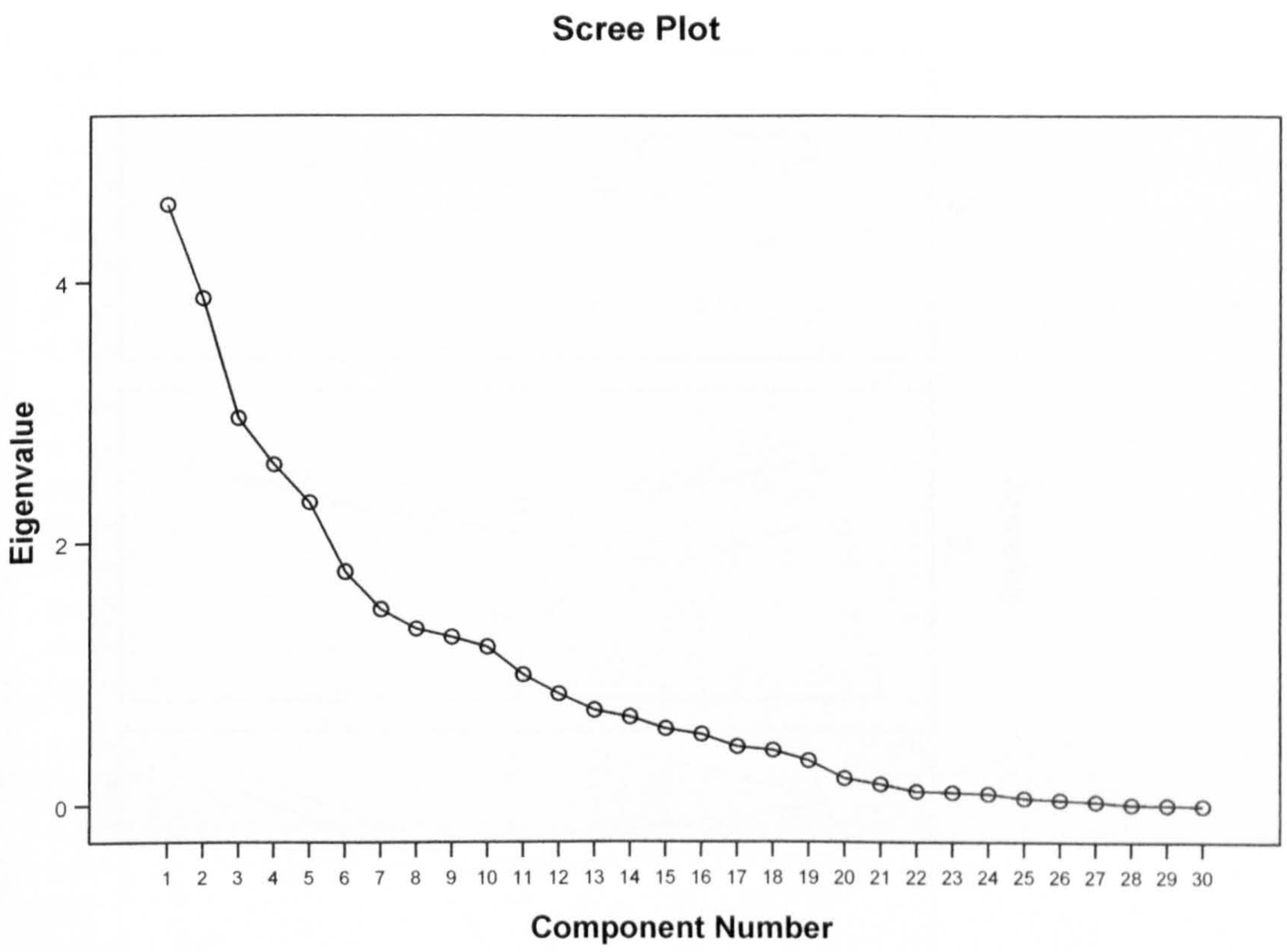


Figure 5.3.5 Scree test plot of net hoof growth/wear rate (mm/day) all measurements

Because the factor analysis results showed unclear loading on 11 components, it was decided to retain all the net hoof growth/wear rate (mm/day) raw data for further investigation using general linear model, mixed between-within subjects analyses of variance. The full net hoof growth/wear (mm/day) data set can be found in Appendix 5.

5.3.11 General linear model analysis of the effects of month and hoof wall location on net hoof growth/wear rates (mm/day)

A mixed between-within subjects analyses of variance was performed on the net hoof growth/wear rate (mm/day) results to analyse the impact of hoof wall location and month of sampling on the net hoof growth/wear rate (mm/day). There was a significant interaction between month and location (Figure 5.3.6), Wilks Lambda = 0.790, F (8,298) = 4.666, $p < 0.001$, partial eta squared = 0.111. Using the commonly used guidelines proposed by Cohen (1988): 0.01 = small effect, 0.06 = moderate effect, 0.14 = large effect, this result represents a moderate effect size.

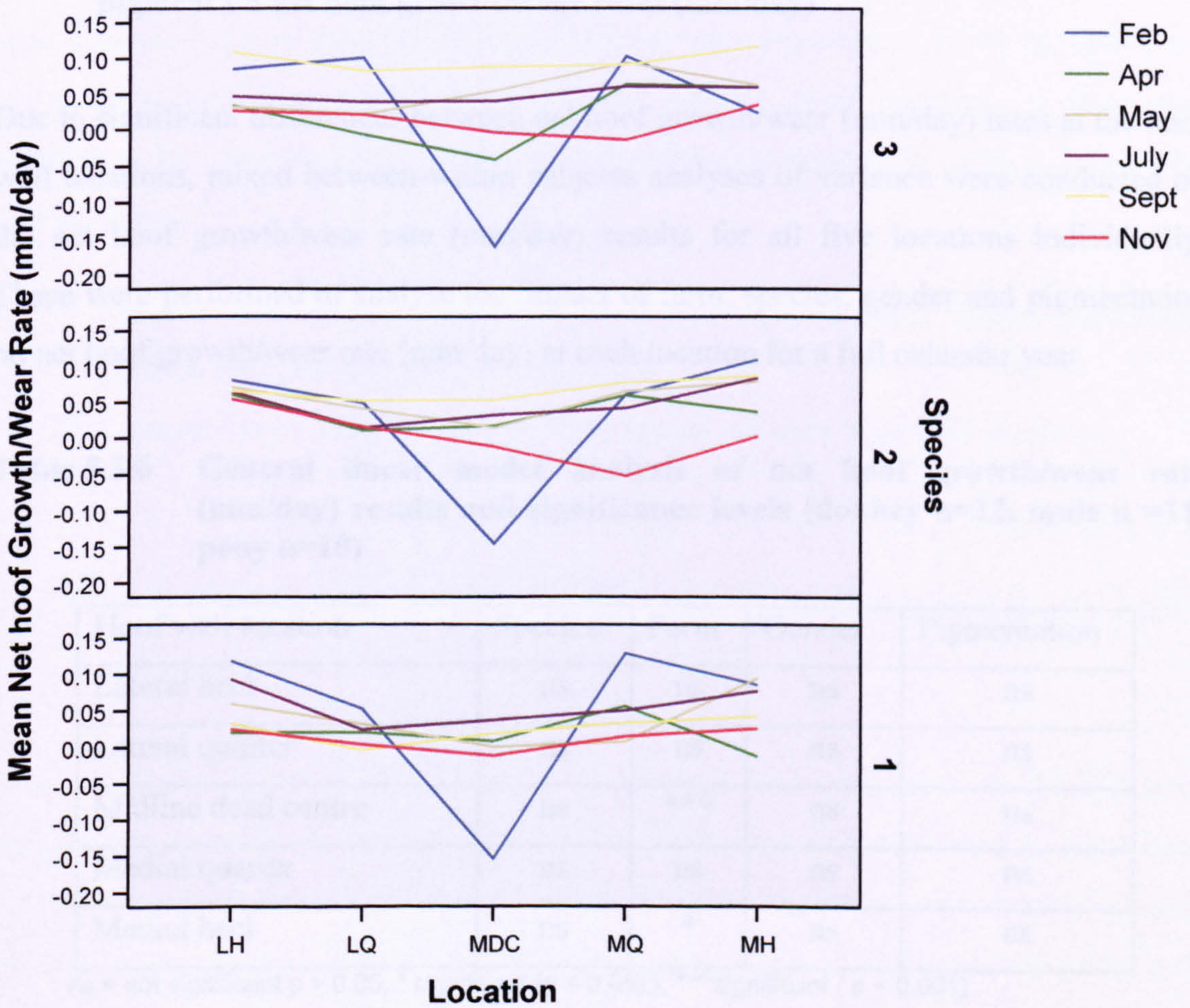


Figure 5.3.6 **Mean net hoof growth/wear rate (mm/day) around the circumference of the hoof for all sampling occasions. Species 1 = donkey, 2 = mule, 3 = pony**

The main effect comparing the months was highly significant, Wilks Lambda = 0.609, F (4,149) =23.907, $p < 0.001$, partial eta squared = 0.391, suggesting that the month has a very large effect on net hoof growth/wear rate (mm/day) (Figure 5.3.6). There is a negative net growth/wear (mm/day) for the midline dead centre sampling site for all species in February, and this continues for ponies into April, but the mules and donkeys then enter a period of positive net hoof growth/wear in April.

5.3.12 General linear model analysis of the effects of species, farm, gender and pigment on net hoof growth/wear rates (mm/day)

Due to significant differences between net hoof growth/wear (mm/day) rates at the hoof wall locations, mixed between-within subjects analyses of variance were conducted on the net hoof growth/wear rate (mm/day) results for all five locations individually. These were performed to analyse the impact of farm, species, gender and pigmentation on net hoof growth/wear rate (mm/day) at each location for a full calendar year.

Table 5.3.6 General linear model analysis of net hoof growth/wear rate (mm/day) results and significance levels (donkey n=12, mule n =11, pony n=10)

Hoof wall location	Species	Farm	Gender	Pigmentation
Lateral heel	ns	ns	ns	ns
Lateral quarter	ns	ns	ns	ns
Midline dead centre	ns	***	ns	ns
Medial quarter	ns	ns	ns	ns
Medial heel	ns	*	ns	ns

ns = not significant $p > 0.05$, * significant ($p < 0.05$),), *** significant ($p < 0.001$)

5.3.13 Hoof replacement time (days) analysis results

Overview

Factor analysis was not carried out on the hoof replacement time data as the five hoof wall locations produced a manageable data set which did not require reducing. Mixed between-within subjects analyses of variance were conducted on the hoof replacement time (days) data. Despite it being evident that the replacement time (days) of all three species varied between the hoof wall locations and that the donkey hoof replacement time was the longest, there were no significant interactions ($p > 0.05$) between hoof replacement time (days) and species, farms, gender, hoof pigment, or hoof wall locations. The data are presented in the following logical order to extract all different comparisons:

5.3.14 General linear model analysis of the effect of species, farm, month, gender and pigment on hoof replacement time (days)

5.3.15 Comparison of hoof replacement time (days) between hoof wall locations

5.3.14 General linear model analysis of the effect of species, farm, month, gender and pigment on hoof replacement time (days)

Mixed between-within subjects analysis of variance was conducted on the full hoof replacement time (days) data set. Hoof replacement time (days) showed no significant interaction with ($p > 0.05$) species, farms, gender, hoof pigment, or hoof wall locations for any of the species. It was evident that the replacement time (days) of donkey hoof was longer than that of both pony and mule hoof for all hoof wall locations (Figure 5.3.6), despite no significant interaction ($p > 0.05$) between hoof replacement time (days) and species.

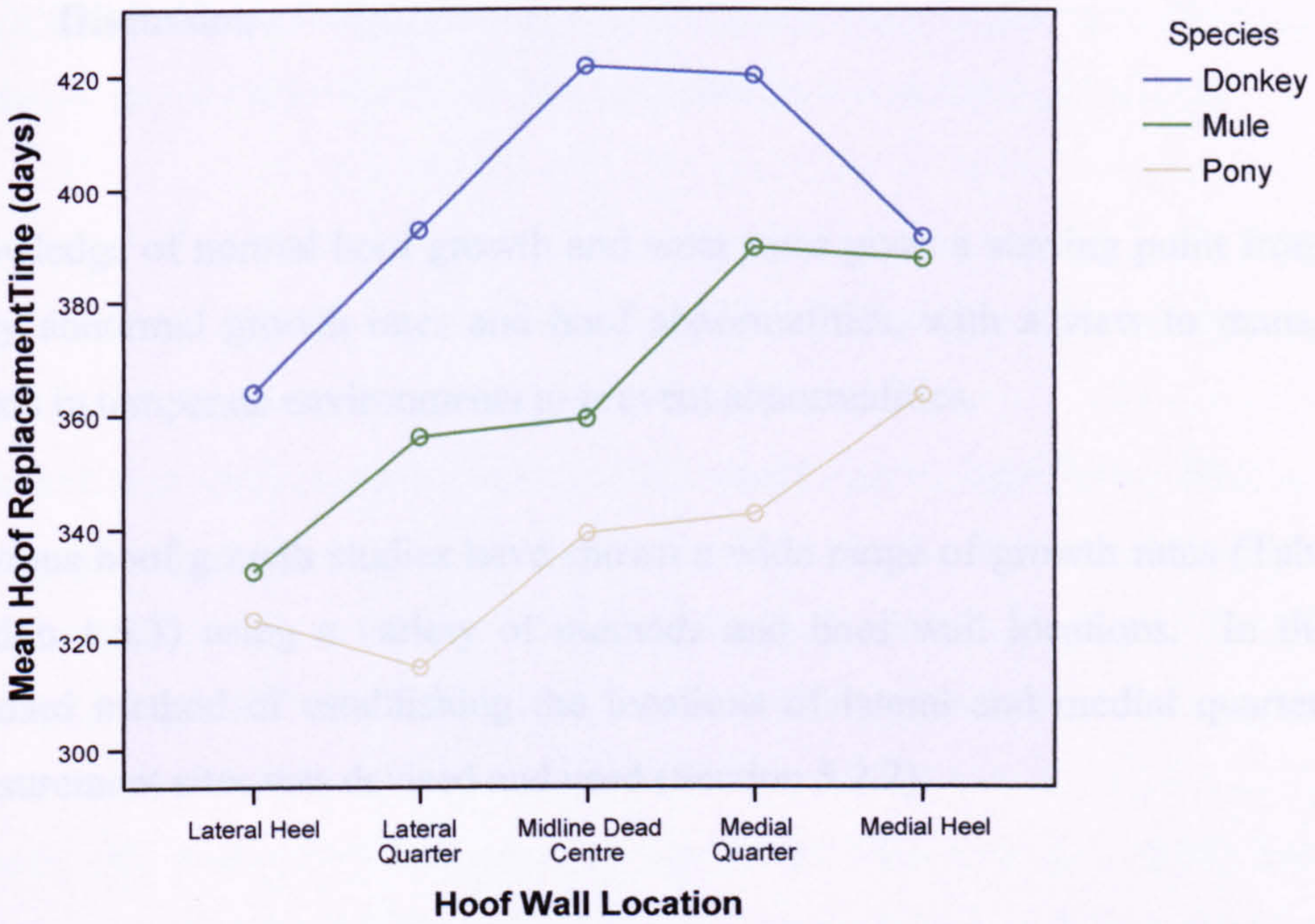


Figure 5.3.7 Hoof replacement time (days) for donkeys, mules and ponies around the circumference of the hoof

5.3.15 Comparison of hoof replacement time (days) between hoof wall locations

Mixed between-within subjects analysis of variance was also conducted on the full hoof replacement time (days) data set to assess interaction between hoof replacement time (days) and the hoof wall location for each of the species. Despite it being evident that the replacement time (days) of all three species varied between the hoof wall locations (Figure 5.3.7), there was no significant interaction ($p > 0.05$) between hoof replacement time (days) and hoof wall location (Table 5.3.7). The donkey hoof replacement time was not only the longest, but it was also the most variable.

Table 5.3.7 Hoof replacement time (days) for donkeys, mules and ponies (n=12 for all species) around the circumference of the hoof

Species	Lateral Heel		Lateral Quarter		Midline Dead Centre		Medial Quarter		Medial Heel	
	Mean	$\pm SE_D$	Mean	$\pm SE_D$	Mean	$\pm SE_D$	Mean	$\pm SE_D$	Mean	$\pm SE_D$
Donkey	363.8	83.24	392.7	86.32	421.7	86.49	420.1	70.26	391.4	73.94
Mule	332.5	65.58	356.0	41.19	359.2	67.91	389.5	66.77	387.5	65.75
Pony	323.7	39.46	314.8	38.36	339.1	67.58	342.4	56.88	362.9	98.48

5.4 Discussion

Knowledge of normal hoof growth and wear rates gives a starting point from which to study abnormal growth rates and hoof abnormalities, with a view to management of equids in temperate environments to prevent abnormalities.

Previous hoof growth studies have shown a wide range of growth rates (Table 1.5.1 in Section 1.5.3) using a variety of methods and hoof wall locations. In this study a standard method of establishing the locations of lateral and medial quarter and heel measurement sites was devised and used (Section 5.2.2).

5.4.1 Hoof Growth Rate (mm/day)

Hoof growth rate (mm/day) results were broadly comparable with the work of other authors, although there was some variability in the data with a total of six standard deviations exceeding ±0.100 SE_D, indicating a broad spread in the data set for those particular dates/hoof wall locations, out of the 90 hoof growth rate results (Appendix 3).

The hoof growth rate figures for pony at the midline dead centre obtained in this study ranged from 0.114 mm/day (±0.046 SE_D) in April, up to 0.200 mm/day (±0.068 SE_D in February. This is comparable to hoof growth figures reported by Reilly *et al.*, (1998a) at midline dead centre in ponies at 0.16mm/day, and also to Reilly *et al.*, (1998b) who found hoof growth rate at midline dead centre in horses to be 0.21 mm/day.

There are no reported mule or donkey hoof growth figures available for comparison, although on the whole the mule midline dead centre hoof growth rate was higher than for both donkey and pony, ranging from 0.157 mm/day (±0.047 SE_D) in November up to 0.221 mm/day (±0.039 SE_D) in September. The donkey midline dead centre hoof growth rates were in general slightly lower than the pony figures, ranging from 0.115 mm/day (±0.078 SE_D) in May, up to 0.165 mm/day (±0.064 SE_D) in February. The

higher rate of hoof growth (mm/day) seen in mules may be due to 'hybrid vigour' as the mules on the trial were the result of a cross between a donkey and a pony.

Effect of farm, gender, pigment, species and month of hoof growth rate (mm/day)

General linear model analysis indicated that there was no significant effect ($p > 0.05$) of farm, gender or pigment on hoof growth rate (mm/day). But month and species had a significant effect ($p < 0.001$) on hoof growth rate (mm/day) (Table 5.3.2). Month alone also had a large effect ($p < 0.001$) on hoof growth rate (mm/day).

In pony and mule hoof there was no significant difference ($p > 0.05$) between hoof growth rates (mm/day) for dates despite the graph (Figure 5.5.3) indicating trends of cyclical growth at alternating sampling occasions. The donkey hoof did not display this trend, but experienced a significant drop in hoof growth rate between September (0.135 mm/day ±0.037 SE_D) and November (0.107 mm/day ±0.031 SE_D). This was the only significant difference ($p < 0.05$) in hoof growth rate (mm/day) between months for any of the species.

Donkeys, mules and ponies displayed the lowest hoof growth rate (mm/day) in the month of November (Figure 5.3.3). This relates to growth in the period between September and November. This drop, which coincides with the onset of colder winter weather, is similar to findings by Bean (1963) in fingernail growth in humans, who reported a 0.03mm/day decrease in fingernail growth when humans were located in the Arctic region, as opposed to the temperate climate of Britain. Josseck *et al.,* (1995) attributed decreased hoof growth rate in winter to fall in temperature, resulting in reduced digital blood flow, which would also account for Bean's (1963) results. MacCallum *et al.,* (2002) reported a decreased rate of horn cell proliferation with decreased day length, and Clarke & Rakes (1982) concluded that cattle hoof growth rates were seasonal and tended to follow variations in daily photoperiod, although this may be an over-generalisation as differences in hoof growth between sampling occasions have certainly been displayed in the present study (Figure 5.3.3). Frackowiak

& Komosa (2006) attributed the decreased hoof growth rate of Konik mares and stallions in winter to a combination of factors including decreased metabolic function, changes in hormonal balance, the drop in day length and ambient temperature.

The reductions in mean hoof growth rate (mm/day) between September and November, for all species, are similar to the 0.03-0.07 mm/day decrease in hoof growth rate found by Geyer and Schultz (1994) during the winter months. Shannon & Butler (1979) also found an autumnal decrease in hoof growth rate in horses over 4 years old (0.23mm/day to 0.19mm/day). Ryan (1989) also found hoof growth rate to be lower in February (0.14mm/day) than in June (0.24mm/day). The decreases in hoof growth rate (mm/day) shown in the current study are in broad agreement with the findings of these authors.

The mules and ponies display cyclical spurts in hoof growth rates, with increased growth rates in February, May and September (Figure 5.3.3). The data for the latter two months could be attributed to seasonal increases in grass growth and therefore nutrient intake. The animals were turned out in spring after the measurements were taken in February. It is known that increased nutritional intake can result in a higher rate of hoof growth (Butler & Hintz, 1977), and an increased level of dietary biotin has been shown to contribute to increased rates of hoof growth (Buffa *et al.*, 1992; Reilly *et al.*, 1998a). Hahn *et al.*, (1986) also found the fastest hoof growth rate in cattle in late spring and early summer. An alternative explanation for increased growth rate is that it is triggered by a preceding period of increased wear (Hahn *et al.*, 1986). This could be a contributing factor in the current trial as the animals were housed in covered straw yards with turnout onto concrete yards for the winter months. This concept and its relevance to results from the current study is discussed in greater detail in Section 5.4.3 and may have relevance for controlled trial design in the future.

Between the species, the mules had the highest hoof growth rate (mm/day) in the spring and summer months, and ponies hoof grew at the highest rate in the autumn and winter months (Table 5.3.2). Both mule and pony hoof had higher hoof growth rates (mm/day) than donkey hoof, and this difference was significant in September and November ($p <$

0.005). This high hoof growth rate (mm/day) in mules surprised the farrier, as he found did not need to trim as much hoof off the mule hooves, so he expected them to have a slower hoof growth rate. This reduced trimming may be explained by the greater wear rate of mule hoof. This concept is explored further in Section 5.4.2.

Donkey hoof growth rate (mm/day) was less than both the other species and if, indeed hoof growth is stimulated by hoof wear, as Hahn *et al.,* (1986) have proposed occurs in cattle, then it may be that the donkeys do not voluntarily travel very far; thus not stimulating the growth that pony and mule hooves produce. The slow growth rate of donkey hooves identified in this study has implications for growing out lesions or other hoof abnormalities, as at the 0.14mm/day growth rate calculated from this study, it would take the average donkey hoof nearly 14 months to grow from the coronary band to the bearing border. This is discussed in greater detail in Section 5.4.4.

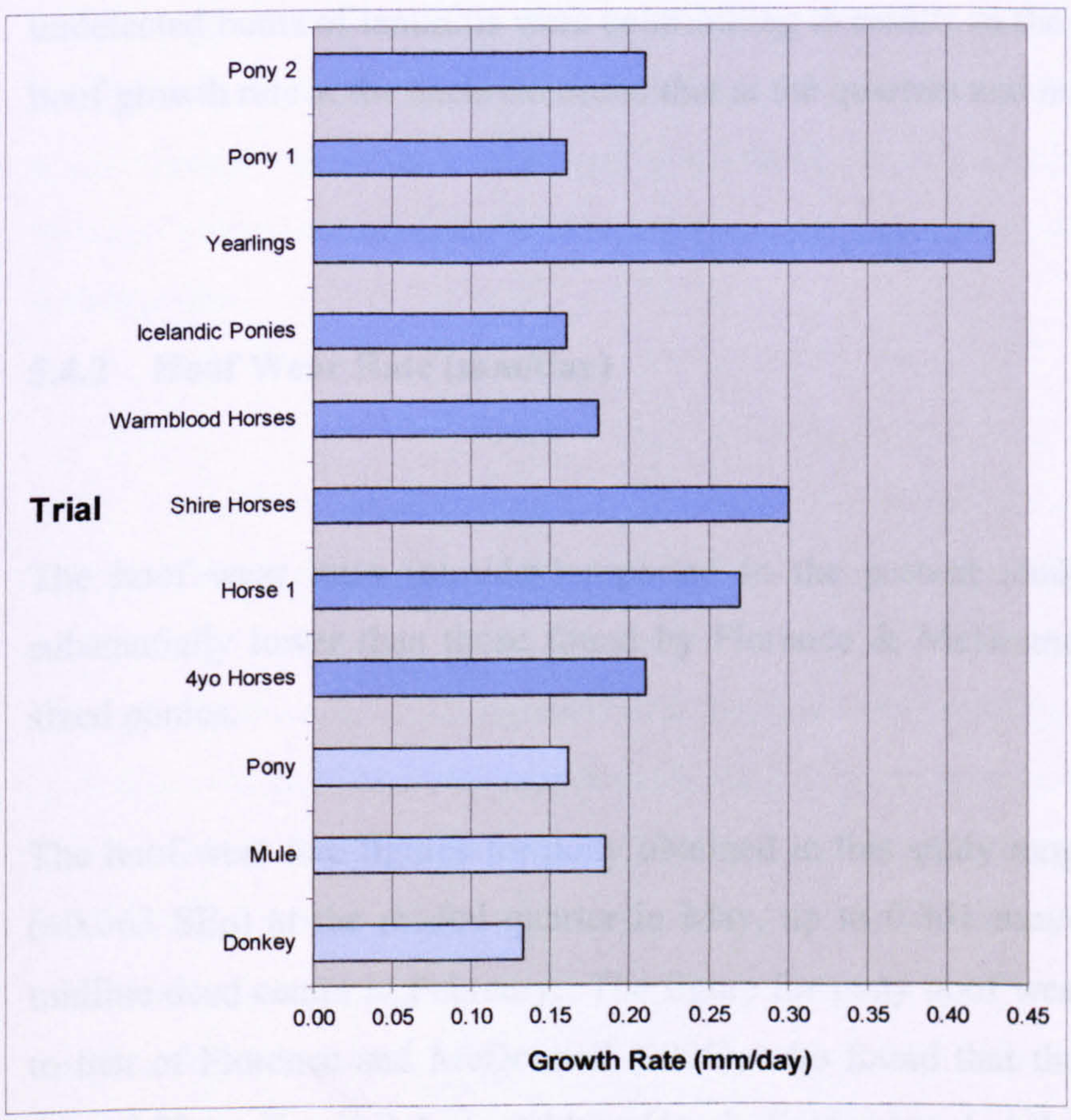


Figure 5.4.1 Equid hoof growth rates at MDC (mm/day)

The growth rates for pony hoof in this study are considerably less than those reported by Graham *et al.*, (1994), but their measurements were of yearling hoof growth, which is known to grow at a considerably faster rate than that of mature animals (Shannon & Butler 1979). Direct comparison with other trials is difficult, as different measurement techniques have been used, but the hoof growth rates of the present study at the midline dead centre location, and those of previous investigations can be seen in Figure 5.4.1. The pony hoof growth rate (mm/day) figure for the present study is very similar to the results of other pony hoof growth trials (Reilly *et al.*, 1998a; Geyer & Schultz, 1994).

Sheta (2002) found an increased rate of hoof growth, particularly at the heels in Arab horses with chronic laminitis, when compared with Arab horses which did not have the disease in a study in Egypt. Similar work could be carried out to establish hoof growth patterns in laminitic donkeys and ponies in a temperate climate. It may be that undetected bouts of laminitis were contributing to results in the present study where the hoof growth rate at the heels exceeded that at the quarters and midline dead centre.

5.4.2 Hoof Wear Rate (mm/day)

The hoof wear rates (mm/day) reported in the present study were, on the whole, substantially lower than those found by Florence & McDonnell (2006) for Shetland-sized ponies.

The hoof wear rate figures for pony obtained in this study ranged from 0.061 mm/day (±0.063 SE_D) at the medial quarter in May, up to 0.361 mm/day (±0.200 SE_D at the midline dead centre in February. The figure for pony hoof wear in May is comparable to that of Florence and McDonnell (2006), who found that the daily wear rate varied from 0.52mm/day in July to 0.24mm/day in September, but the remainder of the pony hoof wear rate (mm/day) figures are no higher than 0.185 mm/day (±0.052 SE_D).

There are no reported mule or donkey hoof wear figures available for comparison, although on the whole the mule hoof wear rate was higher than for both donkey and pony (Appendix 4). The maximum and minimum values do not however, reflect this, as mule hoof wear rates ranged from 0.058 mm/day (±0.039 SE_D) in February at the medial heel, up to 0.353 mm/day (±0.207 SE_D) in February at the midline dead centre. The donkey midline dead centre hoof wear rates were in general similar to the pony figures, ranging from 0.050 mm/day (±0.041 SE_D) at the lateral heel in February, up to 0.319 mm/day (±0.218 SE_D) at midline dead centre in February.

The higher rate of hoof wear (mm/day) seen in mules may be due either to elevated levels locomotion or to some difference in material properties of the mule hoof horn. The mechanical properties of mule hoof have not been investigated, but whilst processing samples in the laboratory for the present study (Chapters 3 and 4) the mule samples appeared more difficult to cut. Whilst obtaining samples for Chapters 3 and 4, the farriers at the Donkey Sanctuary also reported that the mule hoof was more difficult to cut through than both donkey and pony hoof. It may be that there is a correlation between the hardness of the hoof and the rate of wear. This is another area for further investigation.

Effect of hoof wall location on hoof wear rate (mm/day)

General linear model analysis indicated that hoof wall location had a significant effect ($p < 0.05$) on hoof wear rate (mm/day). In all three species the midline dead centre hoof wear rate was significantly higher ($p < 0.05$ to $p < 0.001$) than the quarters and heels (Table 5.3.5). The pony hoof displayed no significant difference ($p > 0.05$) between the midline dead centre and the lateral heel hoof wear rate (mm/day). The break over point for the hoof is at the toe (midline dead centre), and so this area of the hoof is subject to high wear rates which result in the well known 'bevelled edge' (Jackson, 1997) effect at the bearing border of the hoof. No comparative data could be found for hoof wear around the circumference in any of the species in the literature.

Effect of farm, gender, pigment, species and month of hoof wear rate (mm/day)

The general linear model analyses carried out on the wall location hoof wear data produced variable results and did not show any strong trends, with sporadic significant interactions ($p < 0.05$) at various hoof wall locations (Table 5.3.6). It is unclear as to why the medial heel hoof wear rate should produce so many significant interactions with the month, species, farm and gender factors, when the other hoof wall locations did not. The medial heel wear rate is marginally lower than the lateral heel wear rate for both ponies and mules, but the reverse is true of the donkey hoof wear rate, and these differences a not significant. Increased hoof wear rates were seen in cattle which were kept on an abrasive surface such as concrete (Hahn *et al.*, 1986) and also wear rates in cattle were higher in summer than in winter (Clarke & Rakes, 1982) but neither of these trends were seen in the present study, in any species, at any hoof wall location. Florence & MacDonnell (2006) believed that natural hoof trimming coincided with a hardening substrate under-hoof that occurred with the changing climate and season, but the concrete yards that the animals were kept on during the winter months, and the hard, dry paddocks in the summer had no significant effect on the hoof wear rates of any of the species.

Table 5.3.5 shows that the mule hoof wear rate (mm/day) was higher than that of donkeys and ponies at all locations, however the multivariate analysis only produced a significant interaction ($p < 0.001$) between species hoof wear rate (mm/day) and the medial heel hoof wall location. The higher wear rate seen in mule hooves could be due to voluntary movement of the mules being generally higher than that of donkeys and ponies, or greater interaction within the group of mules. Further investigation using pedometers would be required to substantiate this hypothesis.

5.4.3 Net Hoof Growth/Wear Rate (mm/day)

The hoof net growth/wear rates (mm/day) reported in the present study were, on the whole, highly variable when compared to the figures calculated for cattle (Prentice,

1973; Clarke & Rakes, 1982; Hahn *et al.*, 1986: Vermunt & Greenhough, 1990 and Tranter & Morris, 1992). The calculated figures for cattle remain as net growth (positive), ranging from 0.005 mm/day up to 0.056 mm/day, with no reported cases of net wear (negative). The net hoof growth/wear figures in the present study include periods of both net hoof growth and net hoof wear for all three species.

The net hoof growth/wear rate (mm/day) figures for pony obtained in this study ranged from -0.161 mm/day (±0.236 SE_D) at the midline dead centre in February, up to 0.116 mm/day (±0.091 SE_D) at the medial heel in November. Mule net hoof growth/wear rates ranged from -0.146 mm/day (±0.226 SE_D) in February at the midline dead centre, up to 0.110 mm/day (±0.038 SE_D) in February at the medial heel. The donkey net hoof growth/wear rates were, in general, similar to the pony figures, ranging from -0.154 mm/day (±0.248 SE_D) at the midline dead centre in February, up to 0.129 mm/day (±0.088 SE_D) at medial heel in February.

Effect of hoof wall location and month of net hoof growth/wear rate (mm/day)

General linear model analyses of the net hoof growth/wear rate (mm/day) showed significant interactions between month and location ($p < 0.001$) and also a significant main effect for month ($p < 0.001$) on the net hoof growth/wear rate (mm/day). This significant effect of location manifests itself as a sizeable negative net growth/wear (mm/day) for the midline dead centre sampling site for all species in February (Figure 5.3.5).

Hahn *et al.*, (1986) found that the lateral hoof growth/wear rate in cattle was greater than medial hoof growth/wear, which disagrees with the present study for all species in September, and sporadic months for other species throughout the sampling year (Appendix 5), with no significant trend ($p > 0.05$) being seen when comparing the medial and lateral net hoof growth/wear rates. The lack of consistent differences or patterns in net hoof growth/wear (mm/day) in the present study could be due to the high frequency of trimming of the animals in the present trial. This is also a more frequent

trimming regime than is normally carried out on cattle. A properly balanced hoof that strikes the ground flat is more likely to maintain balance (Haight, 2004). The aim of a farrier when trimming the hooves of an equid is to maintain a level hoof which strikes the ground evenly during the stance phase of locomotion. Once the hoof is out of balance, the side with the fastest growth rate will receive more mechanical stress, which may stimulate growth, and therefore continue to grow at a faster rate that the other side of the hoof (Haight, 2004). The trimming and balancing of the hooves essentially reduces and removes any mechanical forces which may have been present due to the hooves becoming unbalanced between trimmings (van Heel *et al.*, 2004).

As can be seen on Figure 5.3.5, the months of February, April and November have the lowest net hoof growth figures for all three species. Net hoof growth/wear rate (mm/day) results for all months and all locations around the circumference of the hoof can be found in Appendix 5.

No sequential monthly data was found for hoof or claw net growth/wear rates in horses or cattle in the literature review with which to compare the present results. Net growth/wear rates have been calculated from growth and wear figures for cattle on difference surfaces, given by a number of authors (Table 5.4.1).

Table 5.4.1 Net Growth/Wear Rate (mm/day) for cattle

Author	Date	Animals	Net Growth/Wear (mm/day)
Prentice	1973	Cows - concrete/pasture Yearlings – straw/pasture Calves – straw/pasture	0.009 0.012 0.054
Clarke & Rakes	1982	Cows – concrete	0.022
Hahn *et al*	1986	Heifers – pasture/dry lot Heifers – concrete	0.035 0.005
Vermunt & Greenough	1990	Heifers – dry lot Heifers – concrete/slats	0.056 0.036
Tranter & Morris	1992	2yo Cows on pasture	0.011

The lowest net hoof growth/wear figures in the present study were seen during a mixture of environments. The February and November hoof growth/wear rates were

recorded on the winter conditions, and the concrete yards that the animals were housed in could have increased the wear rate of the hooves, although there appeared to be no significant effect ($p > 0.05$) of environment on hoof wear rate in Section 5.4.2. Alternatively, as previously suggested in Section 5.4.2, this could be due to a combination of factors including decreased metabolic function, changes in hormonal balance, the drop in day length and ambient temperature Frackowiak & Komosa (2006), or indeed, a lower plane of nutrition.

It was not possible to measure the calorific intake of the animals in the present study, however, it is known that *ad libitum* feed can result in a 50% increase in the hoof growth rate (mm/day) of ponies (Butler & Hintz, 1977). This indicates that further research, to include a record of the calorific intake of the animals involved in the trial, needs to be undertaken in order to establish whether the nutrient intake of the animals at each of the farms has a significant effect on the rate of net growth/wear (mm/day).

The lower net hoof growth/wear rates (mm/day) seen in April may be due either to increased general activity in the spring, or the animals travelling large distances in the paddocks in order to forage for grass.

All species appear to display alternating periods of elevated growth rate (mm/day), followed by elevated wear rate (mm/day), producing cyclical fluctuations in the net hoof growth/wear rates (mm/day) (Appendix 5). Hooves need to grow to ensure that there is sufficient horn to walk on following a period of net wear. Hahn *et al.*, (1986) noted that increased wear preceded increased growth in cattle. For all species the periods of highest net wear were in February and in November, this could be due to the animals being housed, and so their hooves would have been worn on the concrete within the housing areas. Hahn *et al.* (1986), found that heifers confined on concrete only had net hoof growth of 0.005 mm/day, compared to those on pasture which had net hoof growth of 0.035 mm/day but this could have been due to restricted feed intake when confined, rather than access to *ad libitum* grass when at pasture.

Further research is required to ascertain whether the differences in hoof growth are indeed due to preceeding periods of increased wear, or whether they are due to differences in nutritional and calorific intake.

Effect of farm, gender, pigment and species of net hoof growth/wear rate (mm/day)

Because of significant interactions with the hoof wall locations and the net hoof growth/wear rate (mm/day), general linear model multivariate analysis was carried out on the net hoof growth/wear (mm/day) results for all five hoof wall locations individually. No significant interaction ($p > 0.05$) was found between net hoof growth/wear rate (mm/day) and species, gender or pigmentation and any of the hoof wall locations. Specific trials detailing the wear rates of hooves of different pigmentation were not found in the literature review, but the current study is in broad agreement with Benedetti (1948), who found no significant interaction ($p > 0.05$) between the mechanical properties and the pigmentation of the horn. There was also no significant interaction between net hoof growth/wear (mm/day) and the gender of the animals. Frackowiak & Komosa (2006) found the hoof growth rate of mares to be lower than that of stallions, but in their study, the mares were either pregnant or lactating and this has been shown to lower hoof growth rate also in pigs (Simmins, 1985). In the present study none of the females were pregnant or lactating, and all the males were castrated. The mules did not have a significantly higher ($p > 0.05$) rate of net hoof growth/wear (mm/day), despite them having the fastest hoof growth rate (mm/day) (Section 5.4.1). This is because they had a substantial wear rate (mm/day) which lowered the rate of net hoof growth (Section 5.4.2).

The net hoof growth/wear rate (mm/day) interacted significantly with the farm for the midline dead centre ($p < 0.001$) and medial heel ($p < 0.05$) hoof wall locations. It may be that increased locomotion at one farm when compared to another resulted in increased wear rates, leading to lower net hoof growth/wear (mm/day). This is not significant for all locations around the hoof, and further research using pedometers on the animals would be required to substantiate this hypothesis.

5.4.4 Hoof Growth Replacement Time (days)

Despite it being evident that the replacement time (days) of all three species varied between the hoof wall locations (Table 5.3.7), and that the donkey hoof replacement time was the longest (Figure 5.3.6), general linear model analysis showed no significant interactions ($p > 0.05$) between hoof replacement time (days) and species, farms, gender, hoof pigment, or hoof wall locations.

The time taken for the hoof to be completely replaced at the toe in the horse is generally thought to be 9-12 months (Stashak, 2002). Josseck *et al.,* (1995) reported a hoof wall replacement interval of 340 days ±34 days, although the exact corresponding location was not given. The hoof replacement times given at the midline dead centre for mule (359.2 days ± 67.91 SE_D) and pony (339.1 days ± 65.78 SE_D) are largely comparable with the figures given by Josseck *et al.,* (1995) for horse hoof, although the donkey hoof replacement time at midline dead centre (421.7 days ± 86.49 SE_D) is much longer. No equivalent figures were found in the literature review for hoof replacement intervals at the quarters or heels.

All three species displayed longer hoof replacement times for the medial quarter and heel, compared to the lateral quarter and heel hoof locations, although these differences were not significant ($p > 0.05$). This could be due to the medial hoof wall being slightly taller than the lateral hoof wall (Table 5.4.2) in all but the donkey medial quarter hoof wall location.

Table 5.4.2 Mean hoof wall height (mm)

Location	n	Donkey	SE_D	n	Mule	SE_D	n	Pony	SE_D
LH	12	57.04	1.96	12	61.45	2.84	12	63.49	2.87
LQ	12	63.79	1.30	12	70.39	2.20	12	67.49	3.01
MDC	12	65.05	1.30	12	71.48	2.42	12	65.38	2.53
MQ	12	62.12	2.32	12	75.01	2.89	12	74.10	3.47
MH	12	60.29	3.48	12	68.37	3.76	12	68.20	3.63

Even though donkey hoof wall height is lower than the pony or mule hoof wall height (Table 5.4.3), the slow hoof growth rate (mm/day) means that the donkey hoof replacement interval is longer than both mules and ponies.

This slow growth rate of donkey hooves has implications for growing out lesions or other hoof abnormalities, as many of the donkeys arriving at the Sanctuary require remedial farriery. Further study is required to establish whether feed supplementation or other measures could be introduced to increase the rate of horn production to aid the recovery of donkeys admitted to the Sanctuary, as biotin supplementation has been demonstrated to increase hoof growth rate (Comben *et al.,* 1984 , Buffa *et al.,* 1992 and Reilly *et al.,* 1998a), without increasing the calorific intake in horses.

5.5 Conclusions

- In pony and mule hoof there was no significant difference ($p > 0.05$) between hoof growth rates (mm/day) for between months. Donkey hoof experienced a significant drop in hoof growth rate between September and November ($p < 0.05$)

- The mules and ponies displayed cyclical spurts in hoof growth rates, with increased growth rates in February, May and September. Donkeys, mules and ponies displayed the lowest hoof growth rate (mm/day) in the month of November.

- The mule hoof wear rate was higher than for both donkey and pony at all locations, however the multivariate analysis only produced a significant interaction ($p < 0.001$) between species hoof wear rate (mm/day) and the medial heel hoof wall location.

- In all three species the midline dead centre hoof wear rate was significantly higher ($p < 0.05$ to $p < 0.001$) than the quarters and heels

- General linear model analyses carried out on the wall location hoof wear data produced variable results and did not show any strong trends, although the medial heel hoof wear rate produced many significant interactions ($p < 0.05$) with the month, species, farm and gender factors, when the other hoof wall locations did not.

- There was a significant effect ($p < 0.05$) of location on net growth/wear (mm/day) for the midline dead centre sampling site for all species, which was evident as a high net hoof wear in February

- The months of February, April and November have the lowest net hoof growth/wear (mm/day) figures for all three species. All species appear to

display alternating periods of elevated growth rate (mm/day), followed by elevated wear rate (mm/day), producing cyclical fluctuations in the net hoof growth/wear rates (mm/day.

- No significant interaction ($p > 0.05$) was found between net hoof growth/wear rate (mm/day) and species, gender or pigmentation and any of the hoof wall locations. The net hoof growth/wear rate (mm/day) interacted significantly with the farm for the midline dead centre ($p < 0.001$) and medial heel ($p < 0.05$) hoof wall locations.

- General linear model analysis showed no significant interactions ($p > 0.05$) between hoof replacement time (days) and species, farms, gender, hoof pigment, or hoof wall locations.

- Donkey hoof replacement time was greater than that of both mules and ponies due to the slow hoof growth rate (mm/day) of donkey hoof horn.

- All three species displayed longer hoof replacement times for the medial quarter and heel, compared to the lateral quarter and heel hoof locations, although these differences were not significant ($p > 0.05$).

CHAPTER 6 – DONKEY HOOF HORN HYDRATION AND DEHYDRATION

6.1 Introduction

6.1.1 The use of relative humidity environments to manipulate the moisture content of hoof horn

As previously described in Section 1.3.7, the interaction of water with a biological material can be displayed graphically by a curve called the water vapour sorption and desorption isotherm (Leveque, 1994) using a scale of relative humidity environments.

Relative humidity (RH%) is the ratio of vapour pressure of water in the atmosphere compared to the vapour pressure in the atmosphere that is saturated with moisture at a given temperature (Lovell-Smith & Pearson, 2006). Equilibrium humidity is defined as the relative humidity of the ambient atmosphere that has to prevail in order to cause no net exchange of water. Hygroscopic materials are those that have the ability to absorb, retain or desorb water through changes in humidity (Lawrence, 1995). Hygroscopic materials establish equilibrium humidity with that of the surrounding environment, a characteristic which Johannesson & Janz (2002) named "moisture fixation capacity", and Swarbrick & Boylan (1997) termed "equilibrium moisture content".

The presence of water within the hygroscopic material produces a water vapour pressure on the surface of the material. If this pressure is the same as the water vapour pressure of the ambient atmosphere, then the material has achieved equilibrium humidity with its environment and no water exchange will take place. Any difference between the water vapour pressure of the material and the ambient air produces a water exchange whereby the material will absorb moisture from the environment, or water will be lost to the

environment in the form of vapour, until equilibrium humidity is achieved (D'Arcy & Watt, 1980).

The temperature of the sample, and that of the environment surrounding it will also affect the rate of evaporation or absorption (Pratt 1986), so all sorption/desorption isotherms as the name implies, have to be determined at a given, constant temperature. As the moisture is gained or lost through diffusion, larger thicker samples will take longer to reach equilibrium than smaller thinner samples, so larger samples will need to remain within the humidity environment for a longer period of time than thinner samples, until they attain equilibrium mass.

The isotherm is displayed graphically by plotting a series of weight measurements made at different relative humidities, at a constant temperature (20°C in this case). This produces the curve showing changes in the moisture content of the sample (calculated from mass loss/gain) due to water absorption/desorption as a result of the changing relative humidity at a constant temperature (Figure 6.1.1).

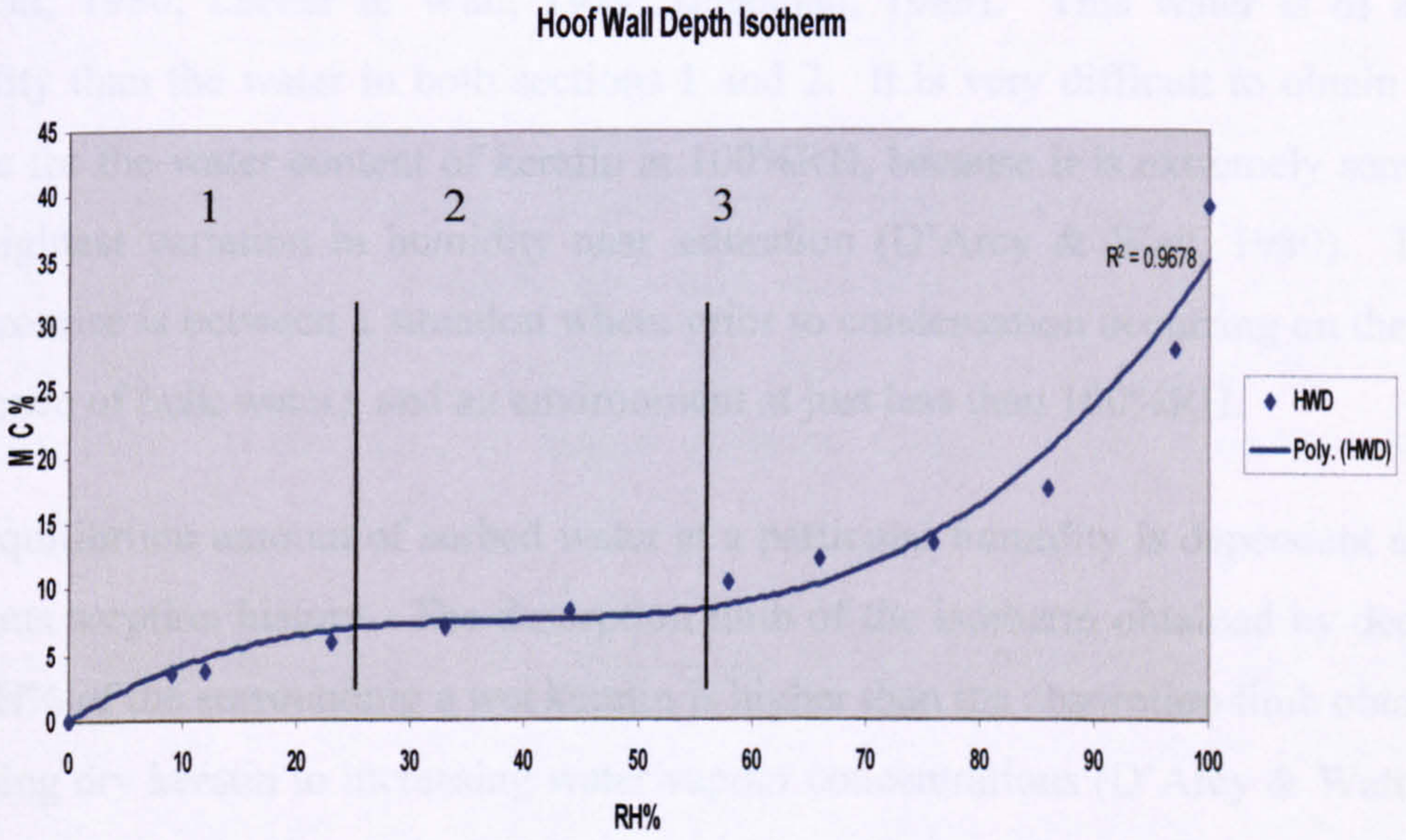


Figure 6.1.1 Water vapour sorption isotherm showing change in MC% at different RH%

The curve of the isotherm represents the interaction of the chemical structure of the material and the water molecules. All isotherms produced for keratinous biological materials tend to be of a sigmoidal shape (Speakman & Cooper, 1936).

Sigmoidal isotherms can be divided into three distinct regions. The first section (1 on Figure 6.1.1) from 0-20RH% is where water is bound to highly active sites, such as charged and highly polar groups. This relates to the amount of strongly bound water molecules in the material studied (Leveque, 1994). The second section (2 on Figure 6.1.1) is a transitional region, where water associates with those sites which are most readily available – such as polar side chains (Bull, 1944). The adsorbed water causes separation of the side chains from the main peptide chains and allows a second layer of water to be adsorbed. The amount of water associated with peptide bonds, which have a low binding energy for water, become proportionately greater with increasing humidity, and at 80% RH accounts for half of the adsorbed water (D'Arcy & Watt, 1980). The third section (3 on Figure 6.1.1) is associated with the condensation of water at very weak binding sites and layering of loosely held water through filling of voids (D'Arcy & Watt, 1980; Leeder & Watt, 1974; Hageman, 1988). This water is of a higher mobility than the water in both sections 1 and 2. It is very difficult to obtain reliable values for the water content of keratin at 100%RH, because it is extremely sensitive to the slightest variation in humidity near saturation (D'Arcy & Watt, 1980). The best compromise is between a situation where prior to condensation occurring on the sample (evidence of bulk water), and an environment at just less than 100%RH.

The equilibrium amount of sorbed water at a particular humidity is dependent upon the previous sorption history. The desorption limb of the isotherm obtained by decreasing the RH% of the surrounding a wet keratin is higher than the absorption limb obtained by exposing dry keratin to increasing water vapour concentrations (D'Arcy & Watt, 1980). This means that the desorption isotherm curve occurs above the sorption isotherm curve (Figure 6.1.2). The curves plotted for sorption and desorption tend to show hysteresis – that is they deviate from one another.

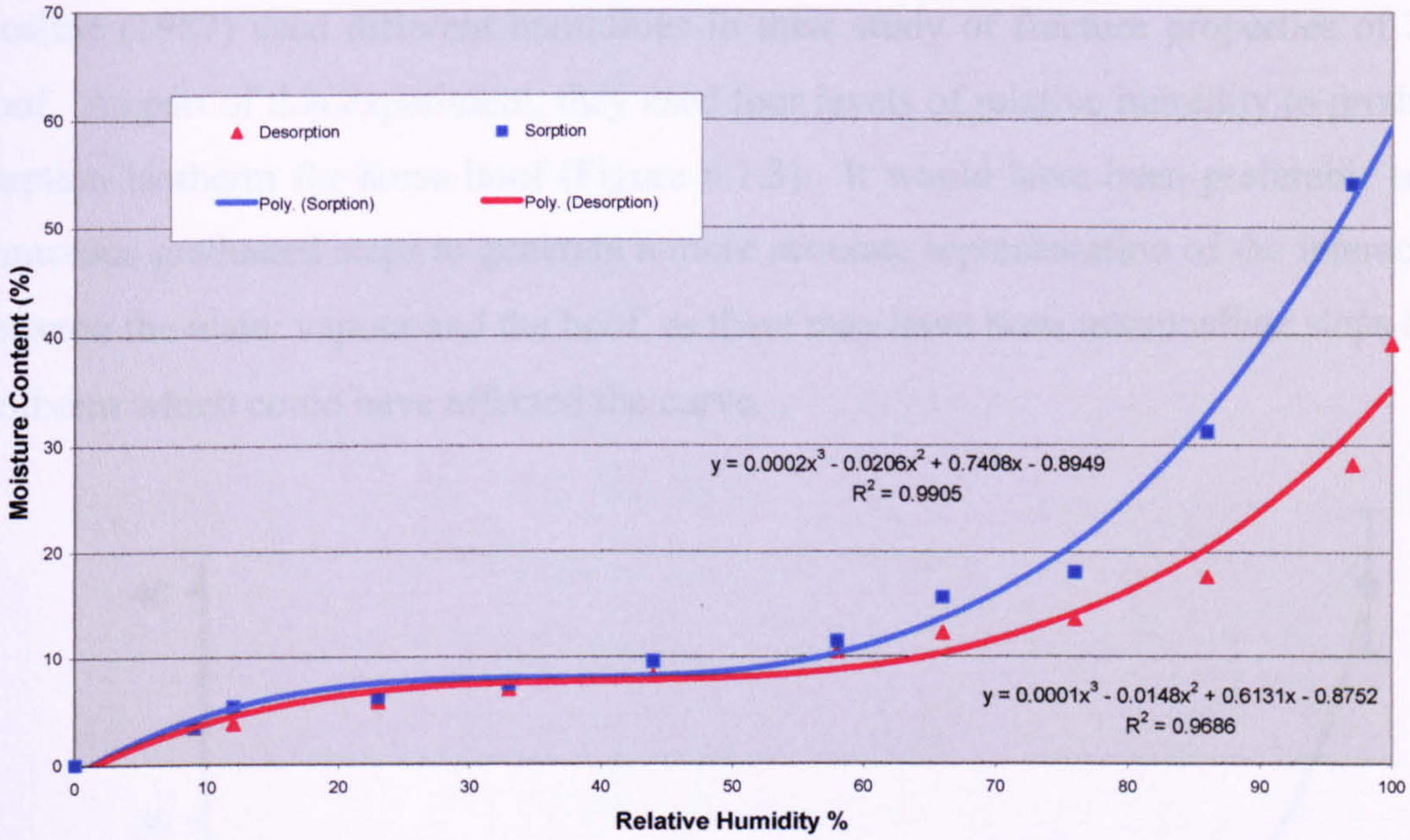


Figure 6.1.2 Water vapour sorption (red) and desorption (blue) isotherms showing the changes in MC% at different RH% and hysteresis effect

D'Arcy & Watt (1980) suggested that hysteresis occurs due to the penetration of the water into the protein structure resulting in swelling because some sites become available for absorption only in the highly swollen state, and these sites continue to bind as relative humidity is lowered. Taylor (1952) hypothesised that the swollen protein network may be unable to collapse in a totally reversible manner. This would mean that more binding sites would be available for water molecules, and so the desorption isotherm curve would occur at higher moisture content values than the absorption isotherm.

The majority of research into the interactions between water vapour and keratins has been carried out by the textile industry, using wool which contains alpha-keratins. These have produced the typical sigmoidal sorption desorption isotherm curves (Speakman & Cooper, 1936) with hysteresis. Reproducible isotherms have also been produced for mohair, horse hair and rhinoceros horn (D'Arcy & Watt, 1980). King (1945) produced a sigmoidal sorption desorption isotherm with hysteresis by conducting a humidity experiment using head horn of unnamed type.

Studies of equine hoof horn water vapour isotherms are rare, although Bertram and Gosline (1987) used different humidities in their study of fracture properties of horse hoof. As part of this experiment, they used four levels of relative humidity to produce a sorption isotherm for horse hoof (Figure 6.1.3). It would have been preferable to use numerous graduated steps to generate a more accurate representation of the interactions between the water vapour and the hoof, as there may have been intermediate steps in the isotherm which could have affected the curve.

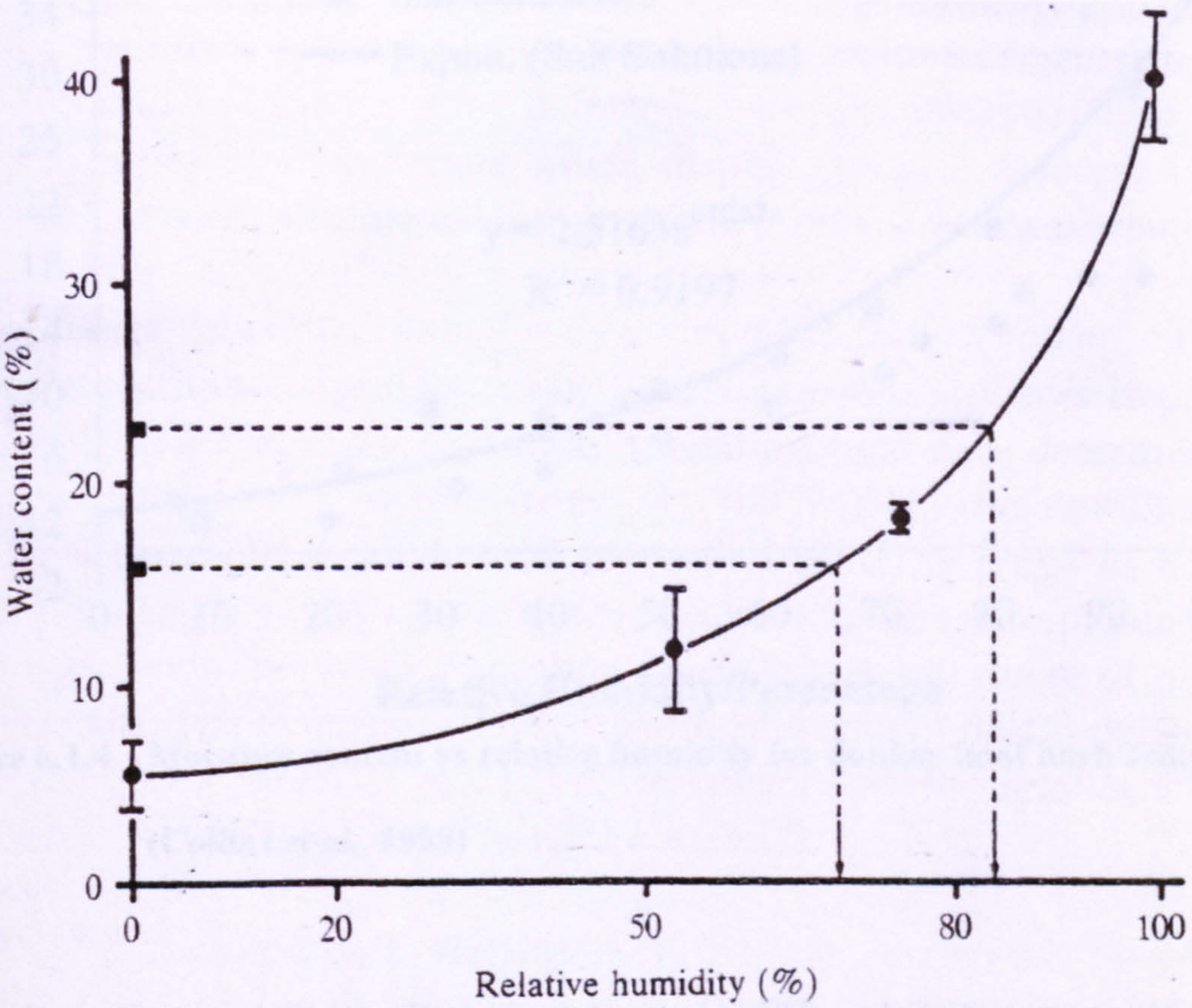


Figure 6.1.3 Absorption isotherm for hoof wall keratin (Bertram & Gosline, 1987)

From unpublished work, Kasapi & Gosline (1997) used extrapolations from that data on sorption isotherms to provide estimated moisture contents for inner, middle and outer hoof wall at 100%RH of 48%, 41% and 35% respectively. Extrapolation is not always reliable as the sorption processes within hygroscopic materials are often complex, so it is safer to record isotherms experimentally.

Collins *et al.,* (1998) plotted moisture content vs relative humidity for donkey hoof samples as part of a investigation into stiffness as a function of moisture content in hoof horn samples. Their graph, while it exhibited hysteresis, did not produce the conventional sigmoidal curve which had been previously described for keratinous materials by Speakman & Cooper (1936) (Figure 6.1.4).

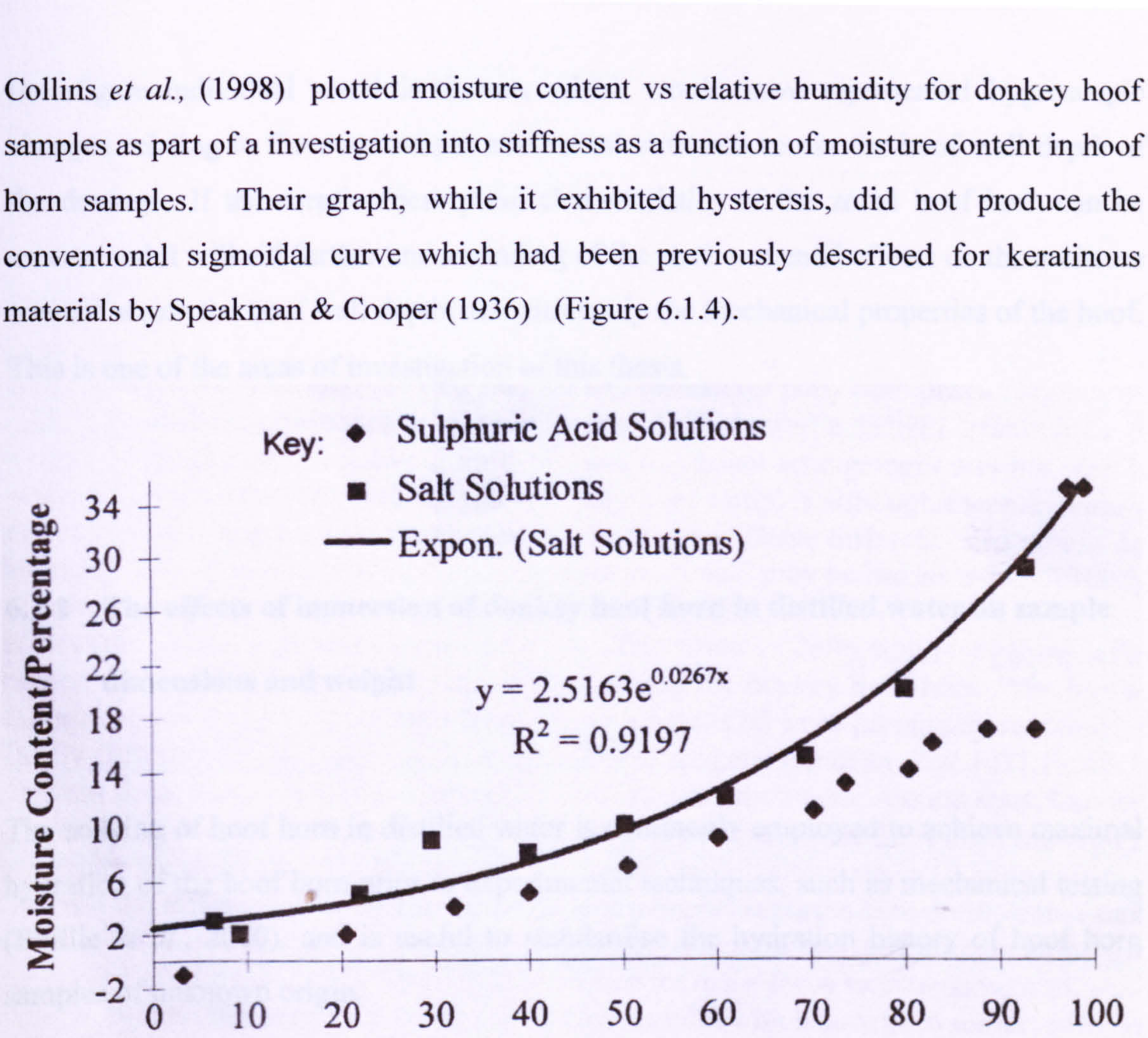


Figure 6.1.4 Moisture content vs relative humidity for donkey hoof horn samples

(Collins *et al.*, 1998)

From the isotherms plotted by Bertram & Gosline (1987) and Collins *et al.,* (1998) it appears that it may be possible that equid hoof horn does not follow the conventional sigmoidal isotherm exhibited by other keratinous materials, including head horn (King 1945). This possibility is one of the areas investigated further in this thesis.

Hopegood (2002) used full hoof wall depth donkey hoof samples to produce a sorption desorption isotherm. These exhibited the characteristic hysteresis between the two curves. The samples used for the sorption curve were different from those used for the desorption curve, which may have an effect on the overall results, as the hoof microstructure will have differed between samples. Hopegood (2002) did not

investigate individual zonal isotherms, which would have represented hygroscopic changes relating to the microscopic anatomical structure across the hoof wall depth of the donkey. If the sorption/desorption characteristics of the zonal hoof horn can be ascertained it will aid further understanding of the environmental effects on the moisture content across the hoof wall depth, and ultimately the mechanical properties of the hoof. This is one of the areas of investigation of this thesis.

6.1.2 The effects of immersion of donkey hoof horn in distilled water on sample dimensions and weight

The soaking of hoof horn in distilled water is commonly employed to achieve maximal hydration of the hoof horn prior to experimental techniques, such as mechanical testing (Baillie *et al.,* 2000), and is useful to standardise the hydration history of hoof horn samples of unknown origin.

Although an investigation into the effects of soaking hoof horn in water has been carried out on horse hoof by Wagner & Hood (2002), serial changes in mass over only 10 days were recorded (Figure 6.1.5) every other day. Subsequently, they also measured osmolarity and Na+, K+ Cl- and protein concentrations of the immersion solution. No record was made of the change in dimensions of the hoof horn samples over the soaking period.

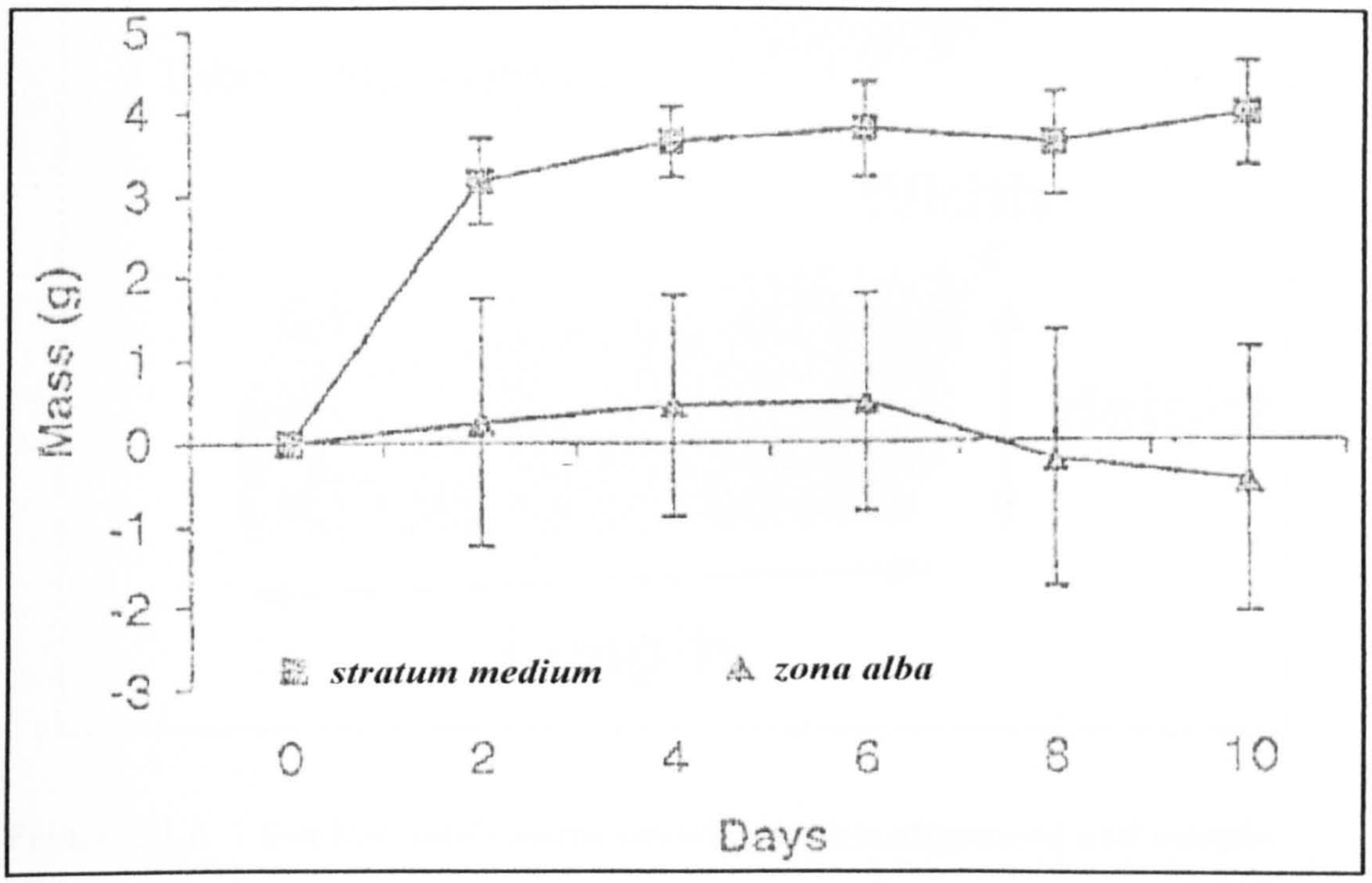


Figure 6.1.5 **Changes in mass of the outer *stratum medium* (squares) and the *zona alba* layer of the *stratum medium* (triangles) of hoof tissues of 10 horses during a 10-day immersion period in water (Wagner & Hood, 2002)**

No studies on the dimensional effects of soaking hoof horn from any species have been found in the literature review for this thesis, although Kreplak *et al.*, (2001) found that the human hair cuticle swelled by 10% following water sorption.

The effects of *in vitro* soaking on the dimensions of zonal and full hoof wall depth samples can be measured in three planes; height which is parallel to hoof tubule alignment; width at right angles to hoof tubule alignment; and length, which corresponds to hoof wall depth (See Figure 6.1.6).

Any increase in the height measurement corresponds to an overall hoof wall height increase as a result of water absorption. Width measurement increase would correspond to an increase in the hoof wall circumference as a result of water absorption. An increase in overall hoof wall depth as a result of water absorption would correspond to an increase in sample length.

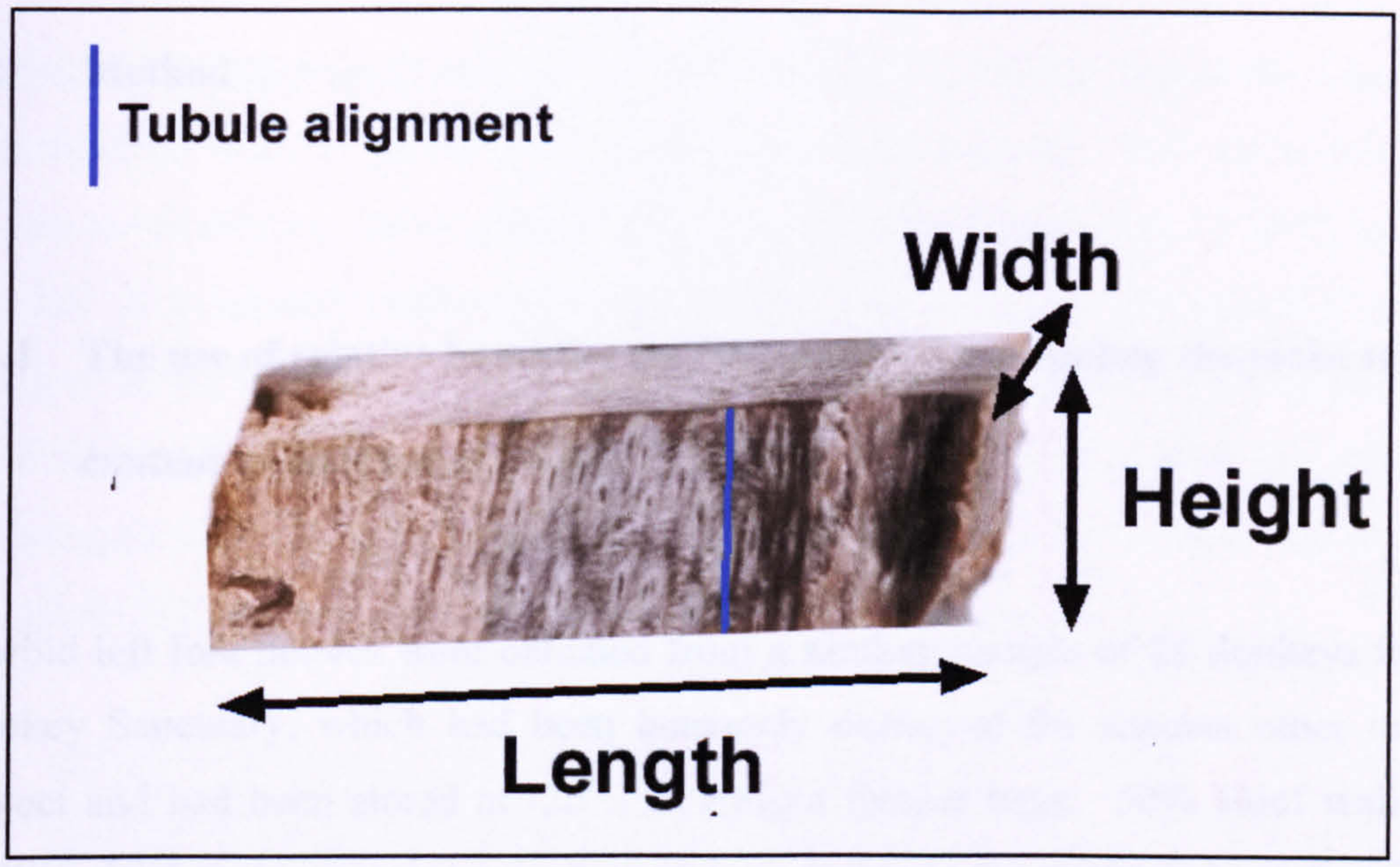


Figure 6.1.6 Donkey hoof sample showing tubule alignment and sample measurements

6.1.3 Aim

The aim of this part of the study was therefore to:

- establish desorption and sorption isotherms for the four zones of the *stratum medium* of donkey hoof horn
- determine mass and dimensional changes over time, for the four zones of the *stratum medium* of donkey hoof horn, when immersed in distilled water.

6.2 Method

6.2.1 The use of relative humidity environments to manipulate the moisture content of hoof horn

Morbid left fore hooves were obtained from a random sample of 21 donkeys from the Donkey Sanctuary, which had been humanely destroyed for reasons other than this project and had been stored at -20°C in airtight freezer bags. 50% Hoof wall height midline dead centre samples were prepared according to the methods in section 2.1.2 to produce four zonal and one hoof wall depth sample per donkey.

The salts type relative humidity cabinet (Ref 413. Copely Instruments (Nottingham) Ltd) was prepared by making up a solution of potassium sulphate, to give relative humidity of 97% (Table 6.2.1).

Table 6.2.1 Saturated salt solutions & relative humidity scale obtained at 20°C

RH%	Salt
97	Potassium sulphate
86	Potassium chloride
76	Sodium chloride
66	Sodium nitrate
58	Sodium bromide
44	Potassium carbonate
33	Magnesium chloride
23	Potassium acetate
12	Lithium chloride
9	Potassium hydroxide
0	Phosphorous pentoxide

Saturated salt solutions (Table 6.2.1) were obtained by mixing 1kg of the required salt with distilled water to produce a suspension slurry in the tray. The salt solutions were chosen to obtain steps of roughly 10%RH at a constant temperature of 20°C, in order to produce a graduated isotherm (Table 6.2.1). The saturated salt solution was then changed to the next salt solution down/up the RH% scale to obtain the next humidity level required. The correct relative humidity is created throughout the cabinet by directing the internal air over the tray of saturated salt solution located in the base of the chamber (Figure 6.2.1). Air was circulated by an integrated fan.

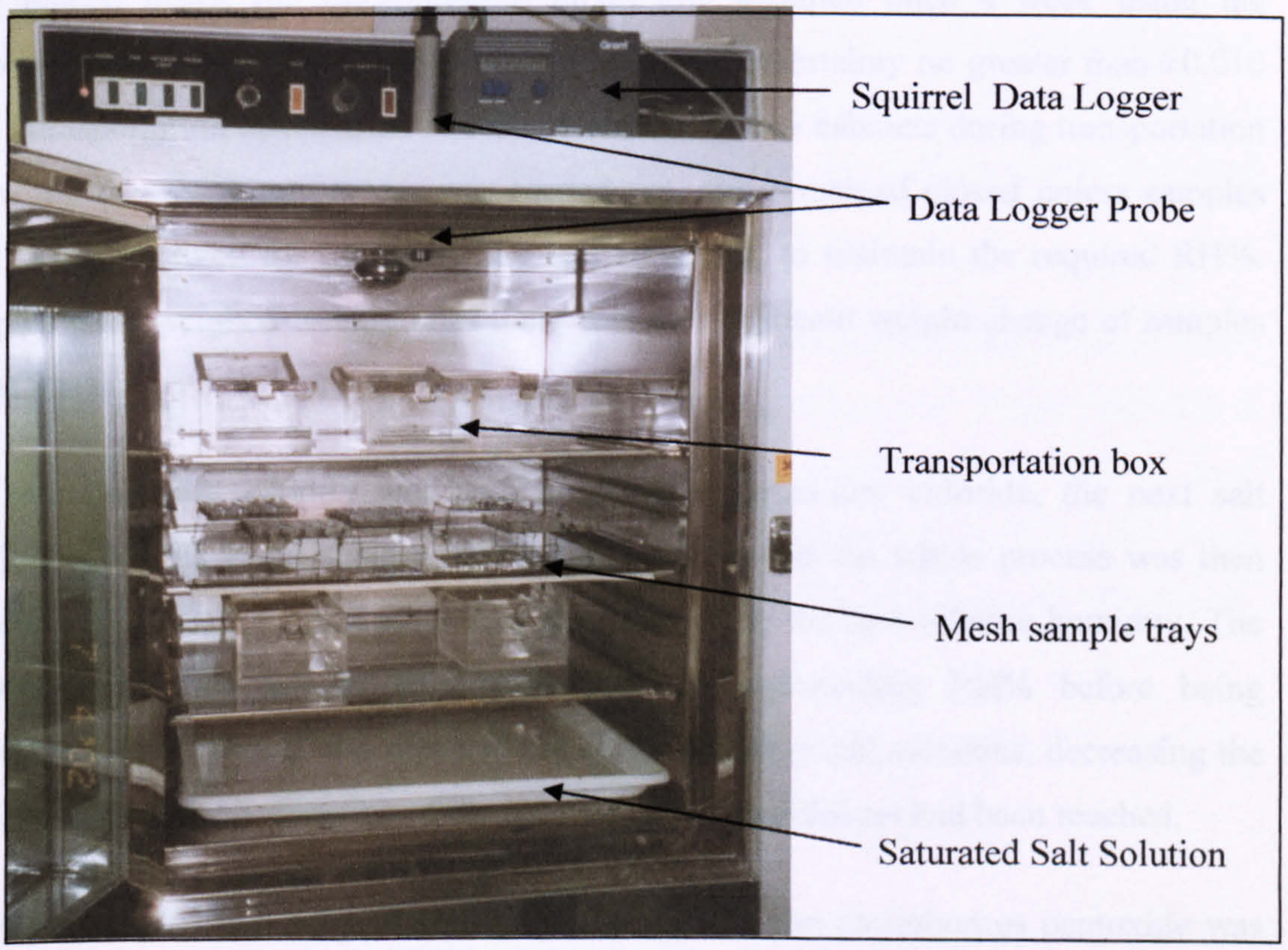


Figure 6.2.1 Relative humidity cabinet

The temperature and relative humidity inside the cabinet were monitored using a Squirrel Data Logger (Figure 6.2.1), which was fitted to a probe inside the humidity cabinet (Grant Instruments Ltd, 1001 series, Cambridge). The cabinet temperature was maintained at 20°C by built-in heater and refrigerator units, which were controlled by a thermostat.

Once the relative humidity had settled at 97%, measured by the probe inside the humidity cabinet, the hoof samples were laid out in the segmented trays and put inside the chamber. The samples were then left to equilibrate with the surrounding RH% until there was no significant change in sample mass. This solution was also placed in the transportation boxes, which were placed, with the lids open, in the cabinet. This ensured that the RH% in the boxes was identical to that in the cabinet.

Samples were then taken from the chamber for weighing on a weekly basis. In order to facilitate weighing and maintain the environmental humidity of the chamber, samples were placed inside the transportation boxes and weighed once a week using the Sartorius balance (Sartorius AG, Germany), with an uncertainty no greater than ±0.010 mg. Transportation boxes acted as mini-relative humidity cabinets during transportation and while the weighing process was carried out, and remained closed unless samples were being removed for (or replaced after) weighing, to maintain the required RH%. Samples were weighed weekly until there was no significant weight change of samples at that RH%.

The saturated salt solution was then changed to potassium chloride, the next salt solution down the RH% scale at 86% (Table 6.2.1), and the whole process was then repeated. The hoof samples were then reintroduced to the new relative humidity. The samples were then left to equilibrate with the surrounding RH% before being reweighed. This process was repeated for all the different salt solutions, decreasing the RH% by 10% at each changing of the solution, once equilibrium had been reached.

Once the samples had reached equilibrium at 0%RH, the phosphorous pentoxide was exchanged for potassium hydroxide (9%RH) and the samples were then rehydrated back up the RH% scale (Table 6.2.1) until equilibrium was reached at 97% with potassium sulphate.

6.2.2 Donkey hoof hydration through immersion in distilled water

Morbid left fore hooves were obtained from a random sample of 8 donkeys from the Donkey Sanctuary. These were then stored and sampled at 50% hoof wall height using the method in Section 2.1.2 and then cut into zonal samples using the method in Section 2.1.4.

These zonal and hoof wall depth samples were then immersed in distilled water and stored at 4°C for two weeks to equilibrate at maximal hydration. Dimensions (mm) and sample mass were determined at 3 hourly intervals during the day. The external surface of each specimen was manually blotted for 2 seconds, to remove surface water prior to mass determination. The specimen was reimmersed in the distilled water following dimension measurements.

The three dimensions, as illustrated in Figure 6.1.5, of each sample were recorded; those dimensions were height, which is parallel to hoof tubule alignment; length, which is at right angles to hoof tubule alignment; and width, which corresponds to hoof circumferential measurement (See Figure 6.1.5). Measurements were made using Mitutoyo Absolute Digimatic callipers (Mitutoyo (U.K) Ltd) to an accuracy of 0.01mm.

6.3 Results

6.3.1 Hoof wall depth sorption/desorption isotherm

The full hoof wall depth sorption and desorption isotherms for donkey hoof horn equilibrated over saturated salt solutions are shown in Figure 6.3.1.

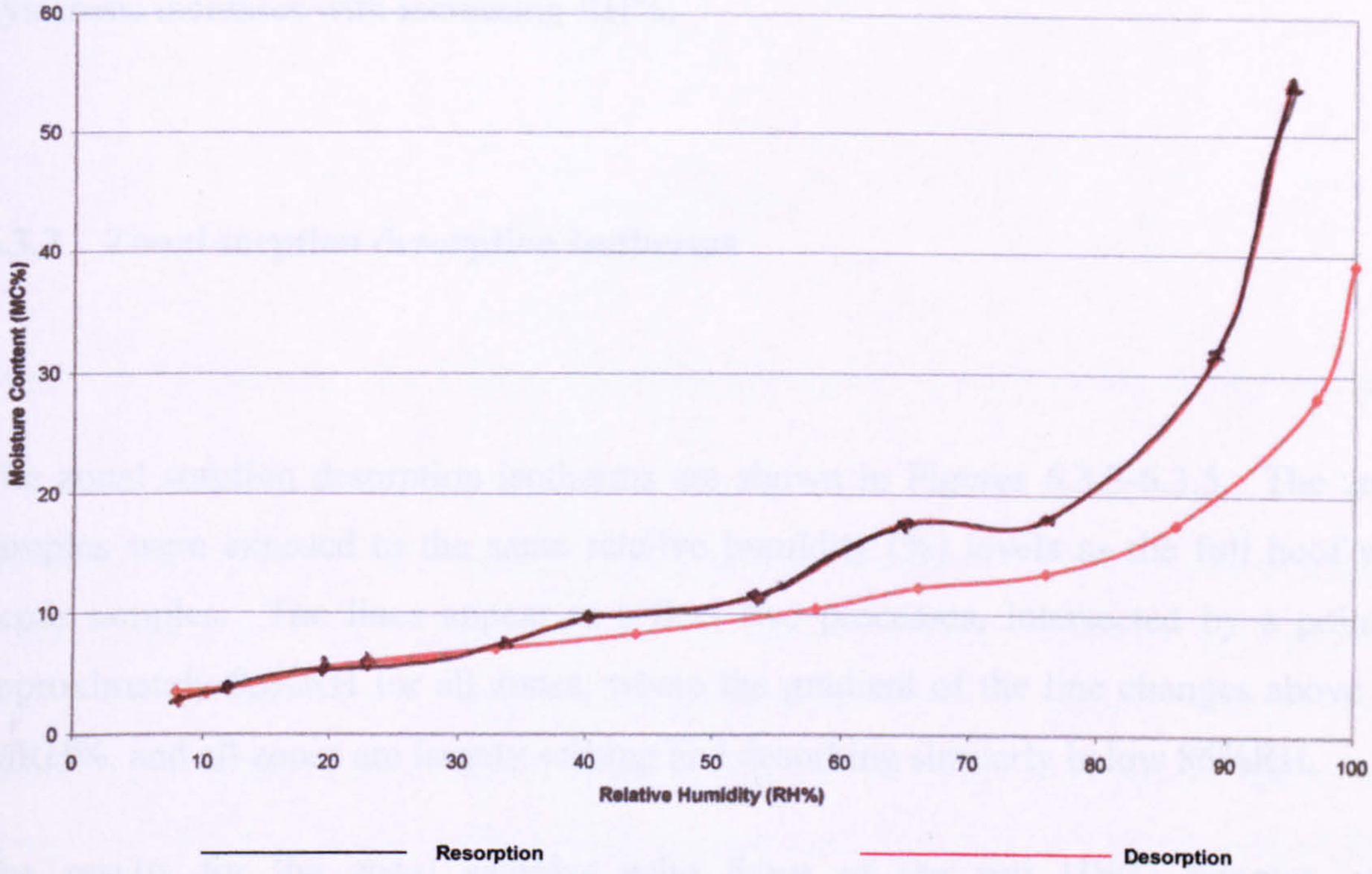


Figure 6.3.1 Donkey hoof horn sorption desorption isotherm full HWD

Samples were left to equilibrate over a minimum period of two weeks. The sample weights at the higher relative humidities of the sorption isotherm took between 3-6 weeks to equilibrate.

The mixed between-within subjects analyses of variance for the full hoof wall depth sorption desorption isotherms showed significant interaction between the relative humidity and the moisture content of the samples, Wilks Lambda = 0.009, $F(10,15) = 165.6$, $p < 0.001$, partial eta squared = 0.991.

In the first section on Figure 6.3.1 from 0-12RH%, where water is strongly bound to highly active sites, both sorption and desorption display extremely shallow almost horizontal lines and there is little hysteresis. In the second transitional region where water associates with the most readily available sites, between 12RH% and 66RH%, both sorption and desorption display slightly steeper lines, but once again there is very little hysteresis. Between 66-86 RH%, both sorption and desorption curves become increasingly steep and the amount of hysteresis. In the third section above 80%, both sorption and desorption lines continue to show increasing steepness, and the amount of hysteresis increases with increasing RH%.

6.3.2 Zonal sorption desorption isotherms

The zonal sorption desorption isotherms are shown in Figures 6.3.2-6.3.5. The zonal samples were exposed to the same relative humidity (%) levels as the full hoof wall depth samples. The lines appear to reflect two processes, intersected by a point at approximately 86%RH for all zones, where the gradient of the line changes above the 86RH%, and all zones are largely sorbing and desorbing similarly below 86%RH.

The results for the zonal samples echo those of the full HWD samples, with multivariate analysis also showing significant interaction between the relative humidity and the moisture content ($p < 0.001$) for all the zones.

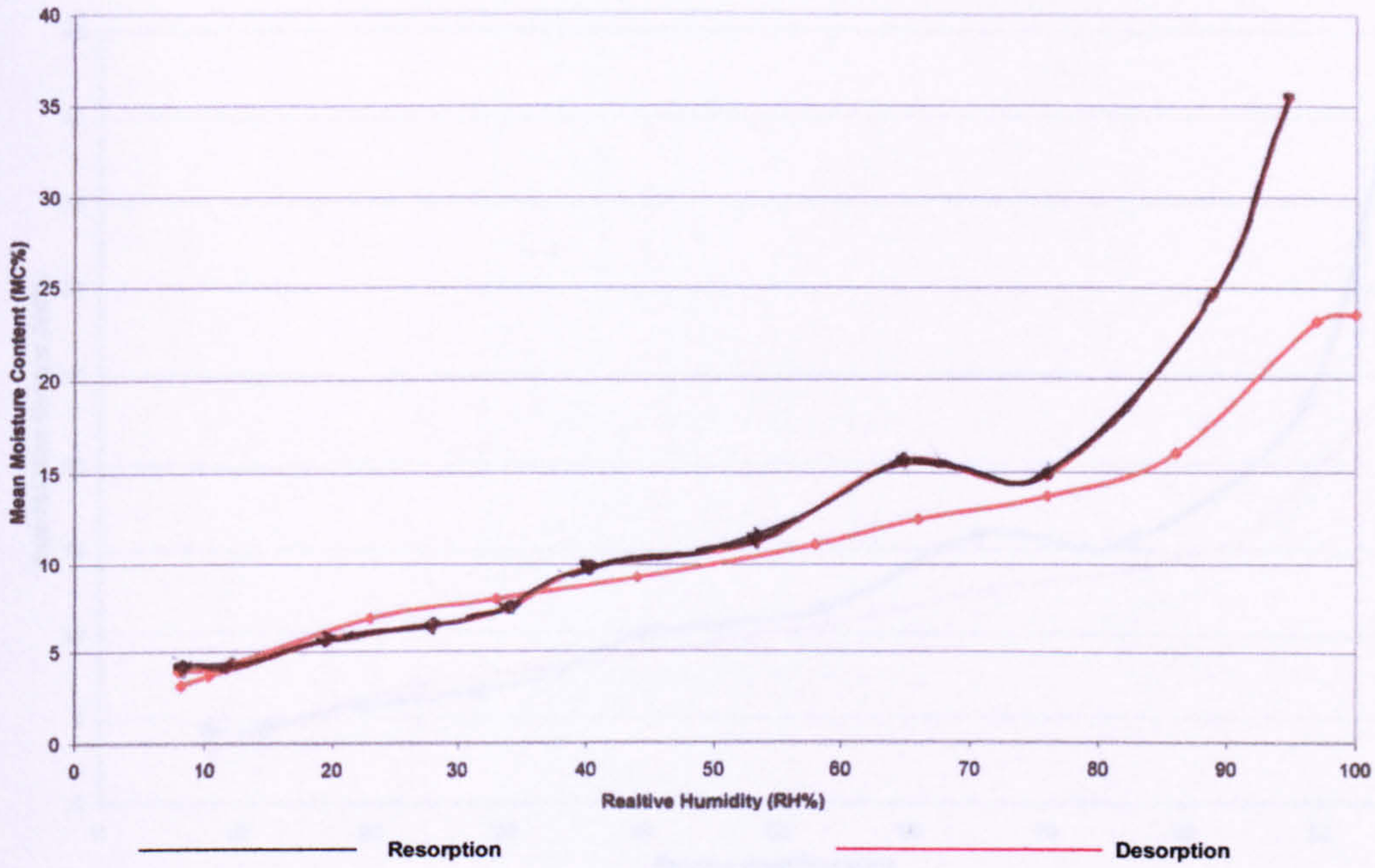


Figure 6.3.2 Donkey hoof horn sorption desorption isotherm for zone 1 samples

As with the full hoof wall depth samples, the first section of the sorption desorption isotherm for zone 1 on Figure 6.3.2 from 0-12RH%, where water is strongly bound to highly active sites, both the sorption and desorption display extremely shallow almost horizontal lines and there is little hysteresis. The second transitional region where water associates with the most readily available sites extends between 12RH% and 86RH% for zone 1. Once again, both sorption and desorption display slightly steeper lines and there is very little hysteresis. In the third section above 86%RH, as was shown in the full hoof wall depth isotherms, both sorption and desorption lines continue to show increasing steepness, and the amount of hysteresis increases with increasing RH%, with a moisture content of 22% for the desorption line and 35% for the sorption line at 100%RH.

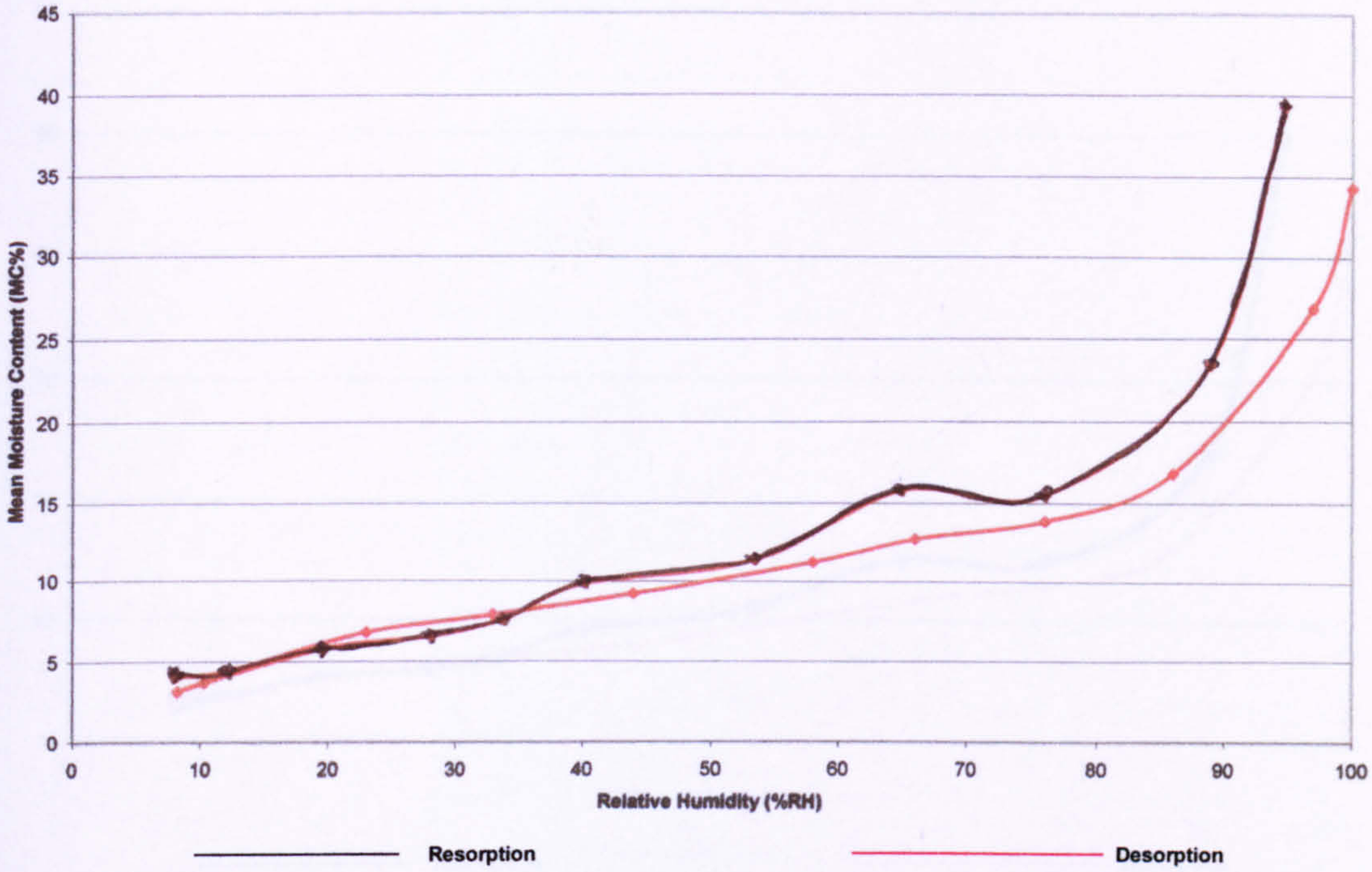


Figure 6.3.3 Donkey hoof horn sorption desorption isotherm for zone 2 samples

Once again, as with the zone 1 samples, the first section of the zone 2 sorption desorption isotherm on Figure 6.3.3 from 0-12RH%, where water is strongly bound to highly active sites, both sorption and desorption display extremely shallow almost horizontal lines and indicate moisture content of around 5%. In the second transitional region where water associates with the most readily available sites, between 12RH% and 86RH%, both sorption and desorption display steeper lines for zone 2 than occurred for zone 1, indicating a higher moisture content % of approximately 15%. In the third section above 86%, both sorption and desorption lines for zone 2 continue to represent increased moisture content compared with zone 1, culminating in a moisture content of between 35 and 40% at 100%RH.

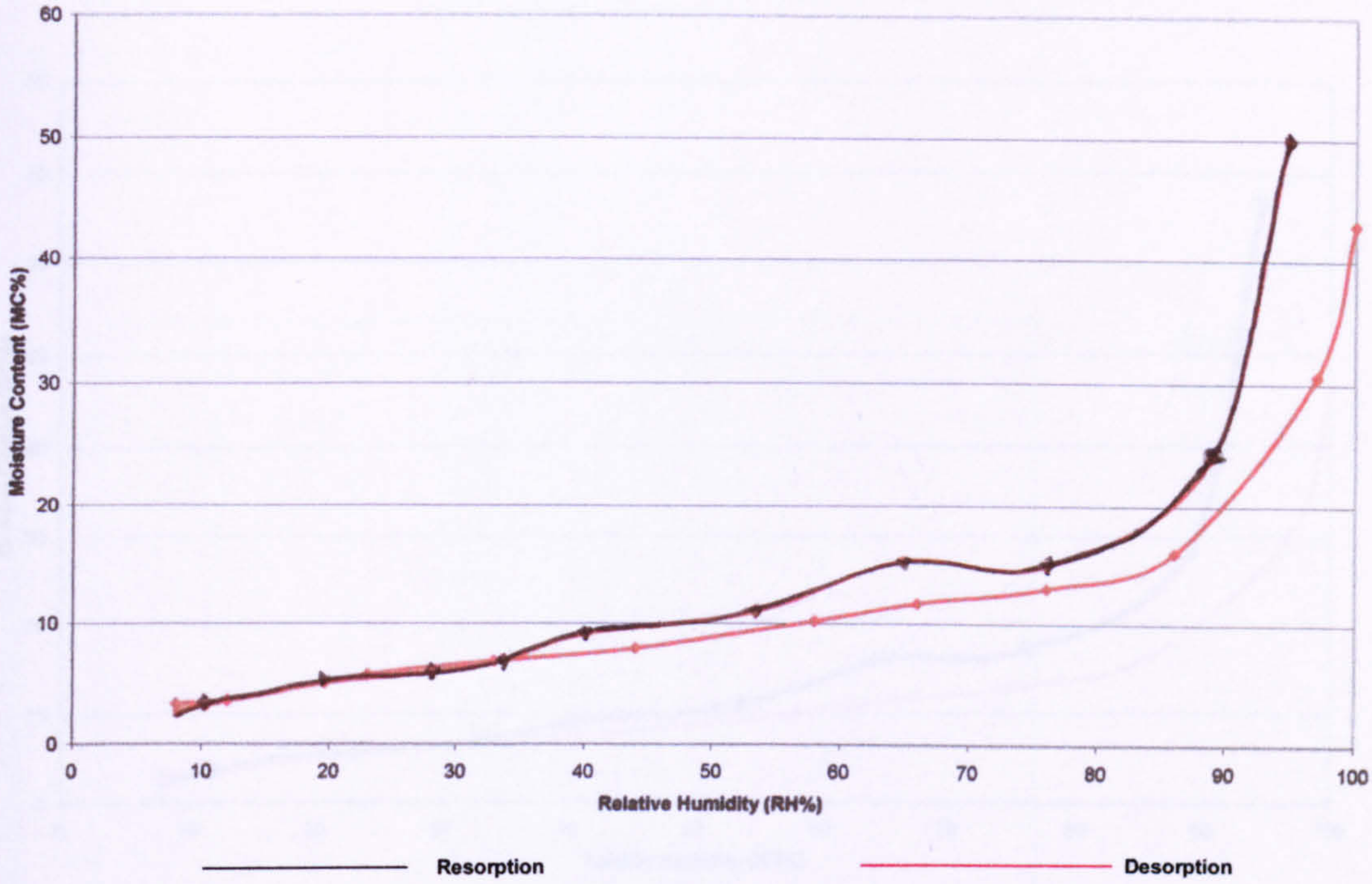


Figure 6.3.4 Donkey hoof horn sorption desorption isotherm for zone 3 samples

The zone 3 sorption desorption isotherm (Figure 6.3.4) displays a shallow line between 8 and 10%RH in the first section and indicates a moisture content of around 3%, which is slightly less than zones 1 and 2. In the second transitional region between 12RH% and 86RH%, both sorption and desorption display steeper lines which are similar to those for zone 2 in Figure 6.3.3, and also indicate a moisture content of approximately 15% at 86%RH. In the third section above 86%, the sorption and desorption lines for zone 3 signify increased moisture content compared with zone 2, which result in moisture contents of 45% for the desorption line and 50% for the sorption line at 100%RH.

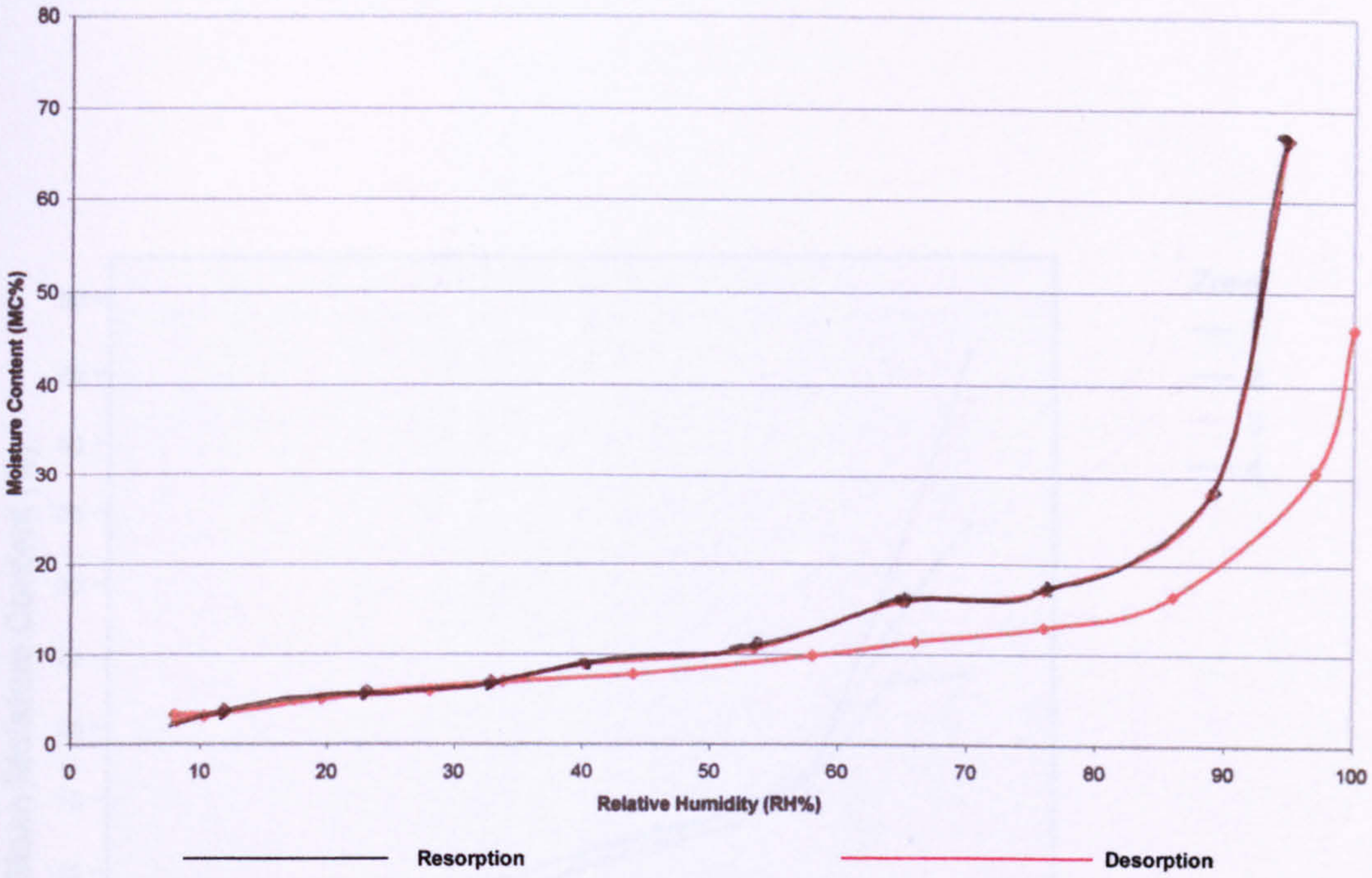


Figure 6.3.5 Donkey hoof horn sorption desorption isotherm for zone 4 samples

The sorption desorption isotherm for zone 4 (Figure 6.3.5) displays a shallow line between 8 and 10%RH in the first section indicating a moisture content of around 3%, which is similar to the zone 3 isotherm. In the second transitional region between 12RH% and 86RH%, also has similar gradient to that seen in the zone 3 isotherm, and also point towards a moisture content of approximately 15% at 86%RH. In the third section above 86%, the sorption line for zone 4 shows a large increase in moisture content compared with zone 3, which gives rise to a moisture content of over 65% for the sorption line at 100%RH, whereas the desorption line is similar to zone 3 at 45% moisture content.

6.3.3 Difference between zonal desorption and sorption isotherms

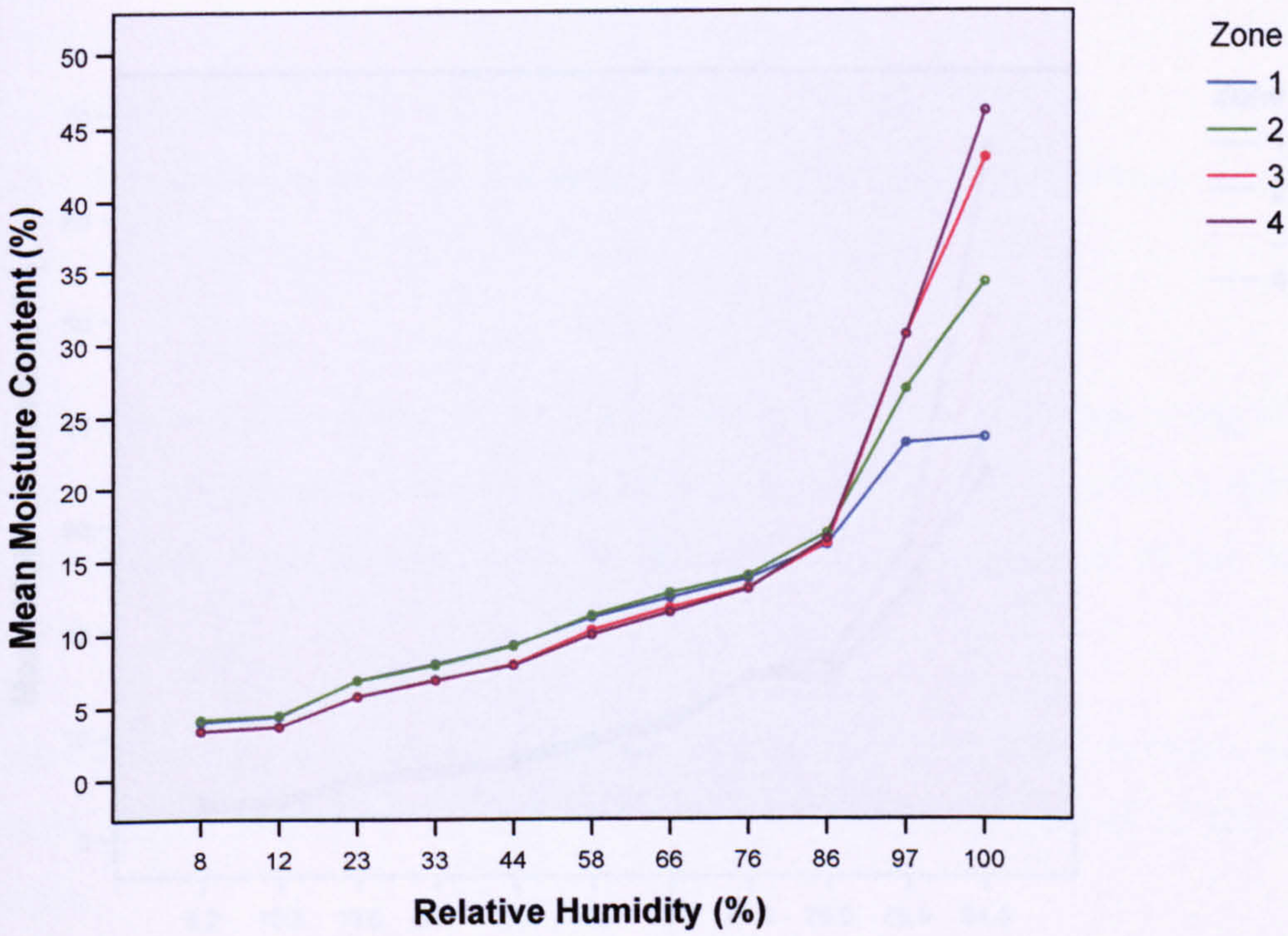


Figure 6.3.6 Donkey hoof horn desorption isotherms for zones 1-4

The zonal desorption isotherm is shown in Figure 6.3.6. For the first section (8-12%RH), and the second section from 12-86RH%, the zones exhibit remarkably similar lines, with the zonal isotherm lines running closely parallel. The diversity in angle of the isotherm line is demonstrated between 86%RH and 100%RH, where difference in the maximal hydration capacity between the zones is evident by moisture contents at 100%RH.

Multivariate analysis of the zonal desorption samples showed significant interaction between the relative humidity and the moisture content ($p < 0.001$) for all the zones, Wilks Lambda = 0.004, $F(10,58) = 1424.5$, $p < 0.001$, partial eta squared = 0.996. There was also a significant difference between the zonal desorption isotherm for zone

1 and the isotherms for the other zones 2, 3 and 4 ($p < 0.005$) which was demonstrated by *post hoc* Scheffe analysis.

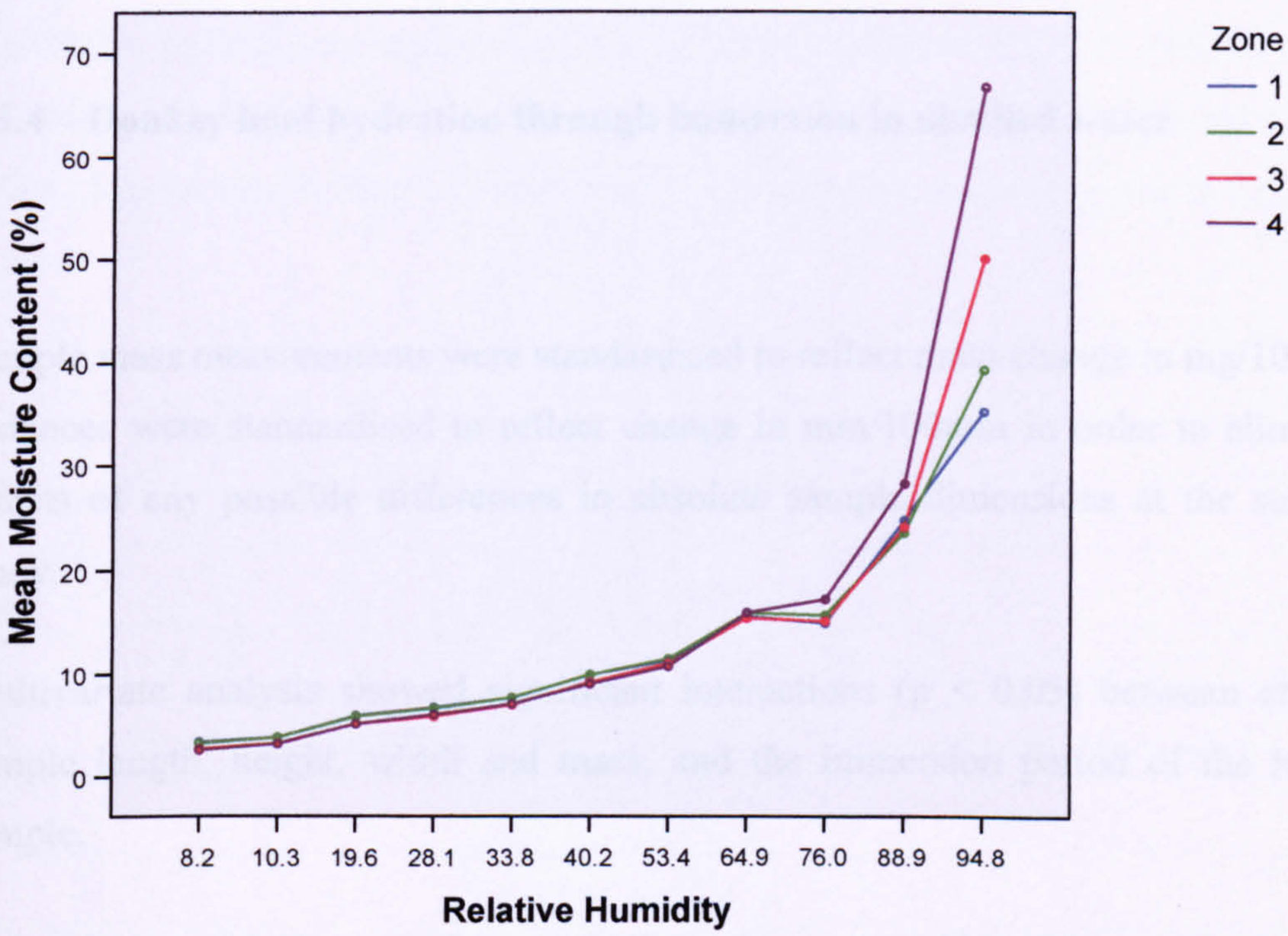


Figure 6.3.7 Donkey hoof horn sorption isotherms for zones 1-4

Similar to the zonal desorption isotherms, the zonal sorption isotherms (Figure 6.3.7) are coincident for both the first (8-12%RH) and the second sections, although the second section appears to finish at 64.9RH% for the sorption isotherms. The diversity in angle of the isotherm line is evident from 64.9% up to 94.8%RH, where the difference in the maximal hydration capacity between the zones is once again evident by variable moisture contents.

As with the desorption, multivariate analysis of the zonal sorption samples showed significant interaction between the relative humidity and the moisture content ($p < 0.001$) for all the zones, Wilks Lambda = 0.003, $F(10,54) = 1617.9$, $p < 0.001$, partial

eta squared = 0.997. There was also a significant difference between the zonal desorption isotherm for zone 4 and the isotherms for the other zones 1, 2 and 3 ($p < 0.005$) which was demonstrated by *post hoc* Scheffe analysis.

6.3.4 Donkey hoof hydration through immersion in distilled water

Sample mass measurements were standardised to reflect mass change in mg/100mg, and distances were standardised to reflect change in mm/100mm in order to eliminate the effects of any possible differences in absolute sample dimensions at the start of the study.

Multivariate analysis showed significant interactions ($p < 0.05$) between changes in sample length, height, width and mass, and the immersion period of the hoof horn sample.

The majority of the increase in sample dimensions was evident 3 hours post immersion, and resulted in a 1% increase in mean sample height, an almost 2% increase in mean sample width and an almost 3% increase in mean sample length. This was reflected in a 3.5% increase in mean sample mass caused by water uptake by the sample in the first three hours.

All data were analysed using SPSS 14 for Windows (SPSS Inc., Chicago, Illinois 60606). The data sets were checked for normality using normal probability plots General Linear Models of repeated measures were used to analyse the data set. Significance for all analyses was assessed at a minimum of $p < 0.05$.

6.3.5 Changes in sample length (mm)

Mixed between-within subjects analyses of variance were conducted on the hoof hydration results, to assess the impact of immersion period and hoof wall zone on hoof hydration in terms of sample length measurement (mm). The main effect comparing the immersion periods was highly significant, Wilks Lambda = 0.237, F (12,17) = 4.556, p < 0.005, partial eta squared = 0.763, suggesting that the immersion period has a large effect on hoof sample length measurement, although this is not apparent in Figure 6.3.8.

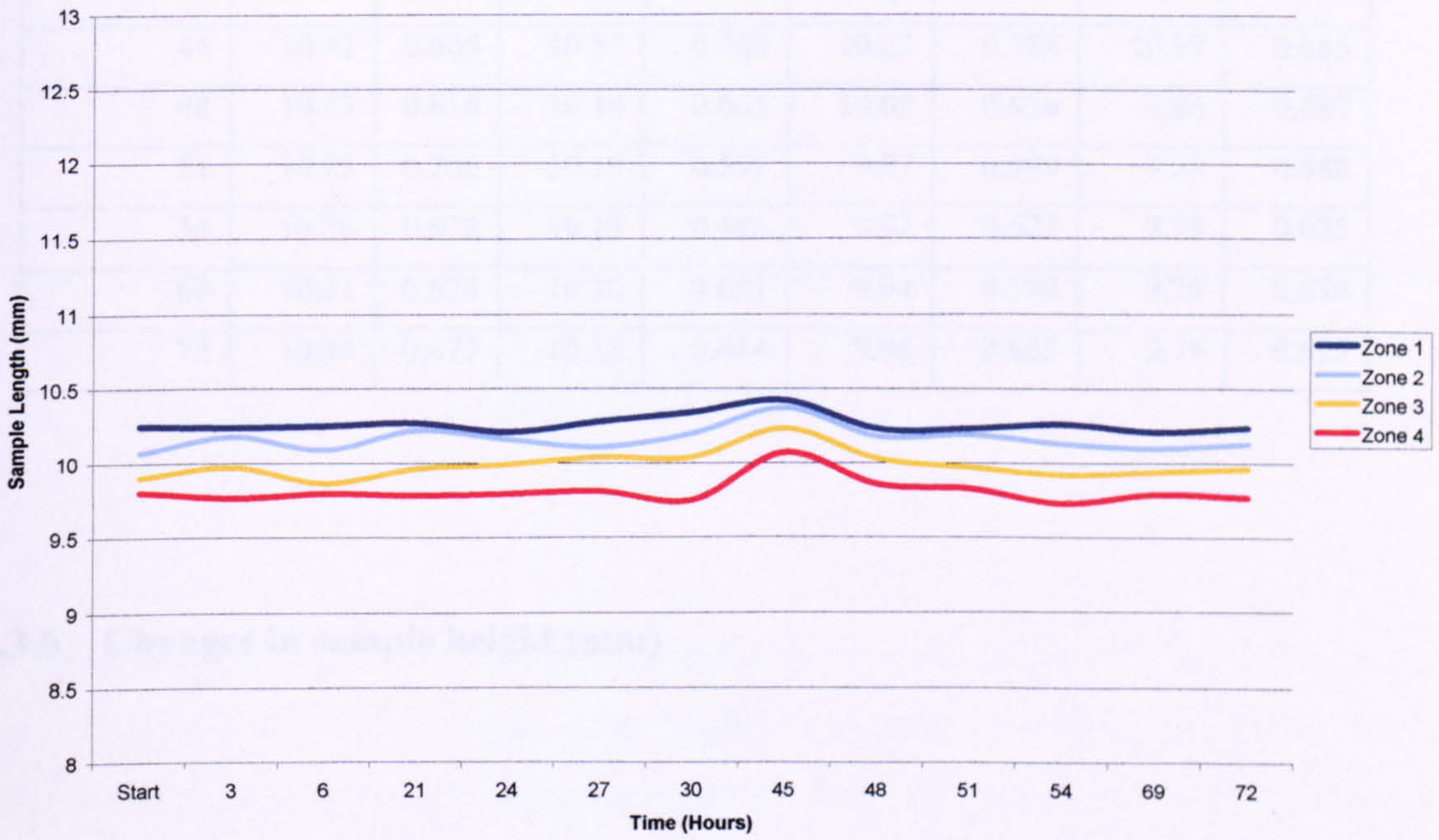


Figure 6.3.8 Fluctuation in length (mm) of zonal *stratum medium* samples of donkey hoof over a 72-hour immersion period in water (n=8 for all zones and HWD)

The mixed between-within subjects analyses of variance for hoof sample length measurements showed no significant interaction ($p > 0.05$) between the immersion period and hoof zone. Absolute sample length measurements are shown in Table 6.3.1.

Table 6.3.1 Mean zonal hoof hydration length (mm) for donkey hoof immersed in distilled water (n=8 for all zones)

Immersion (hours)	Zone 1		Zone 2		Zone 3		Zone 4	
	Mean	$\pm SE_D$	Mean	$\pm SE_D$	Mean	$\pm SE_D$	Mean	$\pm SE_D$
0	10.25	0.626	10.07	0.610	9.90	0.621	9.80	0.669
3	10.24	0.641	10.18	0.685	9.97	0.607	9.76	0.695
6	10.25	0.659	10.09	0.651	9.86	0.555	9.79	0.675
21	10.27	0.601	10.22	0.620	9.96	0..688	9.78	0.738
24	10.21	0.752	10.16	0.601	9.99	0.632	9.79	0.693
27	10.28	0.651	10.11	0.650	10.04	0.630	9.81	0.730
30	10.34	0.635	10.20	0.604	10.04	0.689	9.75	1.962
45	10.42	0.605	10.37	0.545	10.23	0.588	10.07	0.665
48	10.23	0.618	10.18	0.665	10.03	0.636	9.86	0.687
51	10.23	0.706	10.19	0.599	9.97	0.599	9.83	0.688
54	10.26	0.672	10.13	0.666	9.92	0.627	9.73	0.635
69	10.21	0.624	10.10	0.631	9.94	0.598	9.79	0.686
72	10.24	0.677	10.13	0.614	9.96	0.625	9.77	0.675

6.3.6 Changes in sample height (mm)

Mixed between-within subjects analyses of variance were also conducted to assess the impact of immersion period and hoof wall zone on hoof hydration in terms of sample height measurement (mm). The main effect comparing the immersion periods was highly significant, Wilks Lambda = 0.236, F (12,17) = 4.575, $p < 0.005$, partial eta squared = 0.764, suggesting that the immersion period has a large effect on hoof sample height measurement, but this was not evident when the data were plotted Figure 6.3.9.

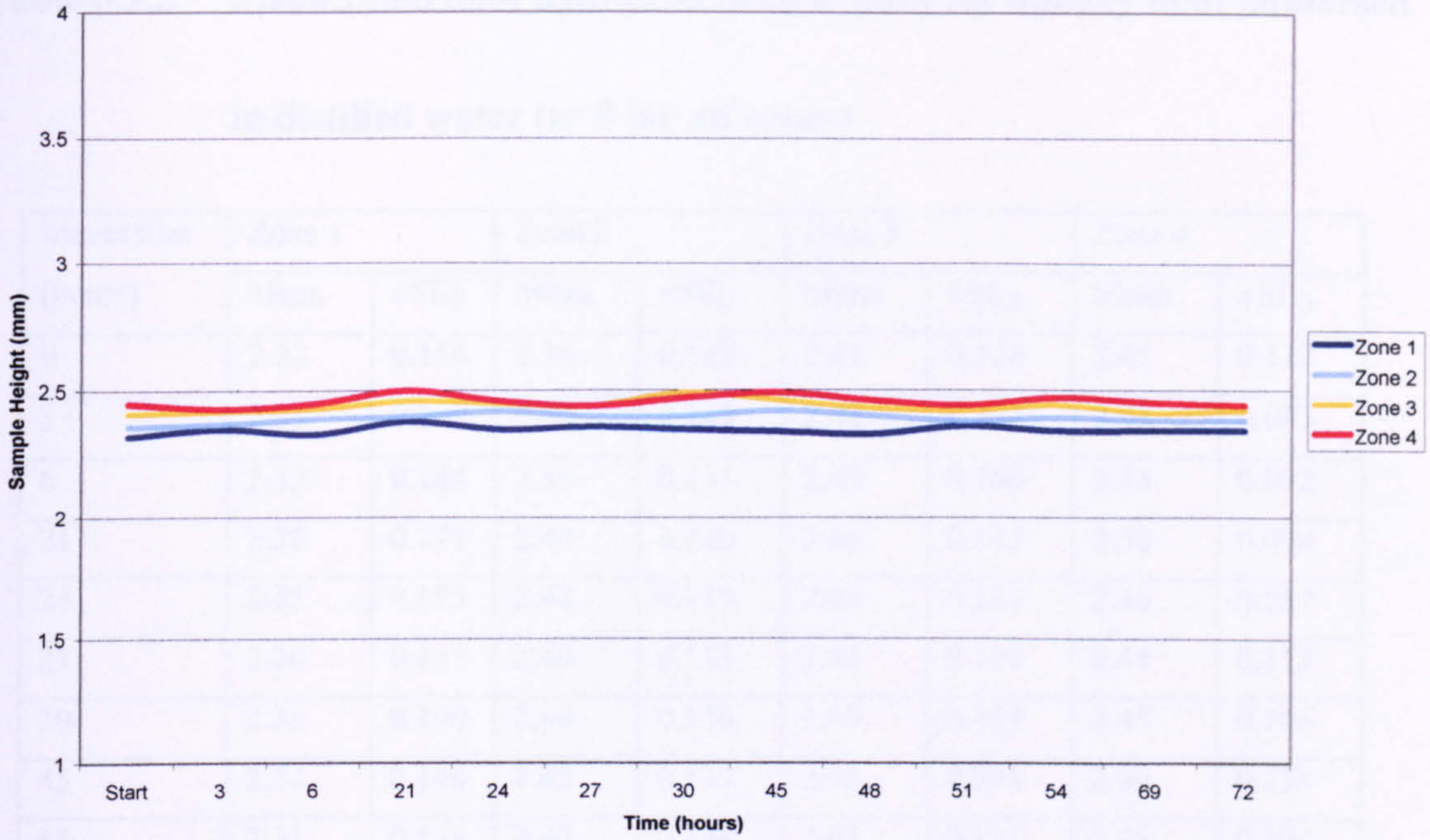


Figure 6.3.9 Fluctuation in height (mm/100mm) of zonal *stratum medium* samples of donkey hoof over a 72-hour immersion period in water (n=8 for all zones and HWD)

Although sample height fluctuated throughout the immersion period for all zones and the full hoof wall depth sample, there were no significant interactions between the sample height changes with immersion period and hoof zone ($p > 0.05$). Absolute sample height measurements are shown in Table 6.3.2.

Table 6.3.2 Mean zonal hoof hydration height (mm) for donkey hoof immersed in distilled water (n=8 for all zones)

Immersion (hours)	Zone 1		Zone 2		Zone 3		Zone 4	
	Mean	$\pm SE_D$	Mean	$\pm SE_D$	Mean	$\pm SE_D$	Mean	$\pm SE_D$
0	2.32	0.156	2.36	0.142	2.41	0.128	2.45	0.112
3	2.35	0.209	2.37	0.148	2.42	0.152	2.43	0.083
6	2.33	0.144	2.39	0.141	2.43	0.166	2.45	0.092
21	2.38	0.171	2.40	0.140	2.46	0.141	2.50	0.094
24	2.35	0.175	2.42	0.119	2.45	0.121	2.46	0.087
27	2.36	0.157	2.40	0.133	2.44	0.109	2.44	0.113
30	2.35	0.190	2.40	0.156	2.49	0.184	2.47	0.106
45	2.34	0.146	2.42	0.135	2.46	0.098	2.49	0.134
48	2.33	0.173	2.40	0.134	2.43	0.131	2.46	0.104
51	2.36	0.164	2.38	0.139	2.42	0.105	2.44	0.067
54	2.34	0.162	2.39	0.129	2.44	0.149	2.47	0.072
69	2.34	0.174	2.38	0.132	2.41	0.111	2.45	0.079
72	2.34	0.168	2.38	0.140	2.42	0.115	2.44	0.0808

6.3.7 Changes in sample width (mm)

Mixed between-within subjects analyses of variance were also conducted to assess the impact of immersion period and hoof wall zone on hoof hydration in terms of sample width measurement (mm). The main effect comparing the immersion periods was highly significant, Wilks Lambda = 0.1089, $F(12, 17) = 14.413$, $p < 0.001$, partial eta squared = 0.911, which suggests that the immersion period has a large effect on hoof sample width measurement although this was not displayed when the data was plotted (Figure 6.3.10).

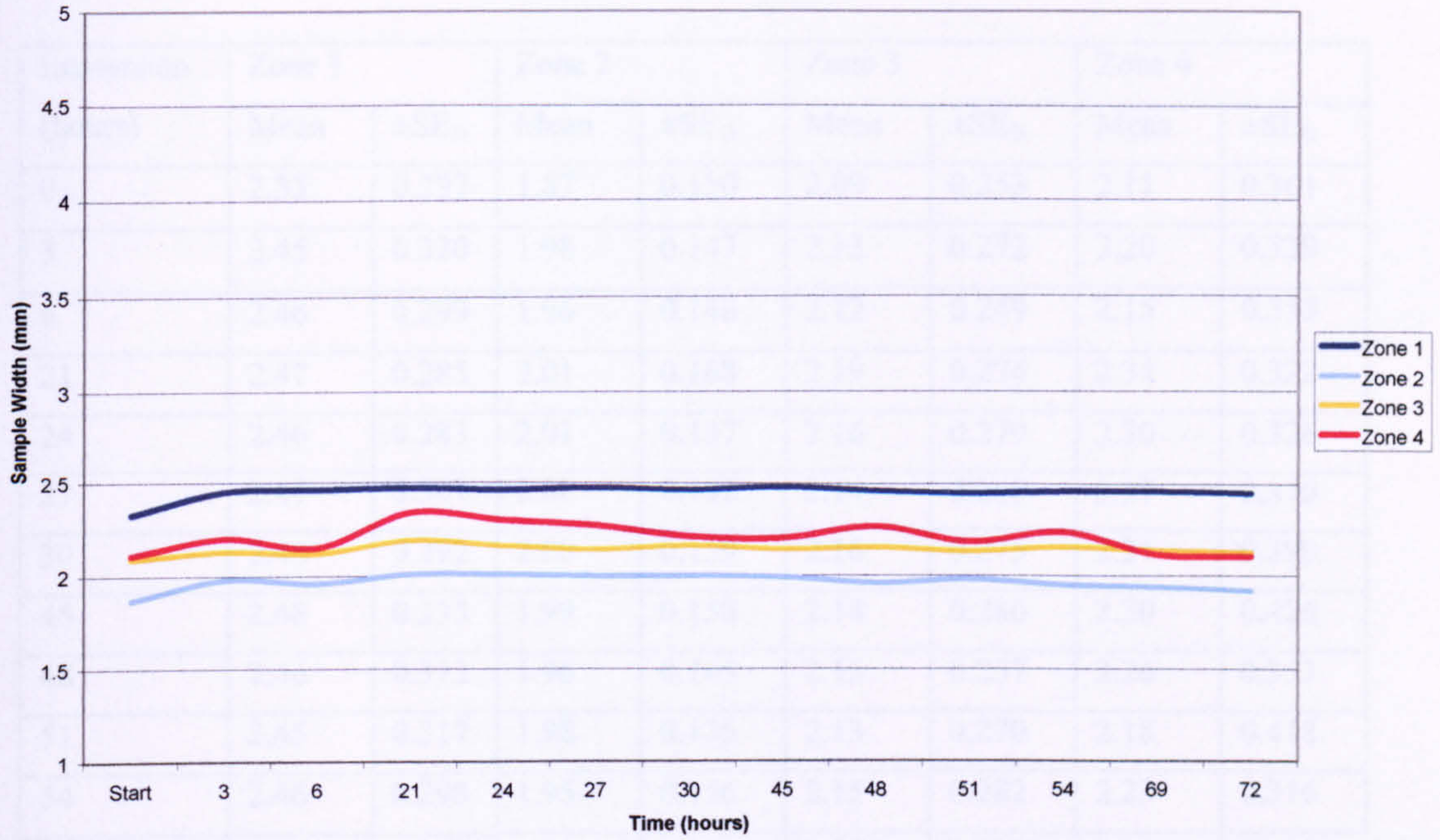


Figure 6.3.10 Fluctuation in width (mm) of zonal *stratum medium* samples of donkey hoof over a 72-hour immersion period in water (n=8 for all zones and HWD)

There was, in addition, a significant interaction displayed between the sample width changes with immersion period and hoof zone, which can be seen in Figure 6.3.10 and Table 6.3.3. Wilks Lambda = 0.100, F (36, 51) = 1.666, $p < 0.05$, partial eta squared = 0.486. Absolute sample width measurements are shown in Table 6.3.3.

Table 6.3.3 Mean zonal hoof hydration width (mm) for donkey hoof immersed in distilled water (n=8 for all zones)

Immersion (hours)	Zone 1		Zone 2		Zone 3		Zone 4	
	Mean	$\pm SE_D$	Mean	$\pm SE_D$	Mean	$\pm SE_D$	Mean	$\pm SE_D$
0	2.33	0.297	1.87	0.150	2.09	0.253	2.11	0.361
3	2.45	0.320	1.98	0.147	2.13	0.272	2.20	0.329
6	2.46	0.299	1.96	0.148	2.12	0.249	2.15	0.355
21	2.47	0.285	2.01	0.168	2.19	0.274	2.34	0.322
24	2.46	0.283	2.01	0.137	2.16	0.279	2.30	0.326
27	2.47	0.301	2.00	0.132	2.14	0.265	2.27	0.370
30	2.46	0.292	2.00	0.159	2.16	0.275	2.21	0.399
45	2.48	0.333	1.99	0.150	2.14	0.280	2.20	0.426
48	2.46	0.312	1.96	0.145	2.15	0.257	2.26	0.357
51	2.45	0.317	1.98	0.136	2.13	0.270	2.18	0.418
54	2.46	0.298	1.95	0.156	2.15	0.282	2.23	0.316
69	2.46	0.312	1.94	0.150	2.13	0.294	2.11	0.406
72	2.45	0.306	1.92	0.160	2.12	0.279	2.10	0.431

6.3.8 Changes in sample mass (mg)

Mixed between-within subjects analyses of variance were also undertaken to assess the impact of immersion period and hoof wall zone on hoof hydration in terms of changes in sample mass (mg). The main effect comparing the immersion periods was highly significant, Wilks Lambda = 0.043, $F(12, 17) = 31.205$, $p < 0.001$, partial eta squared = 0.957, which indicates that the immersion period has a large effect on hoof sample mass measure (Figure 6.3.11).

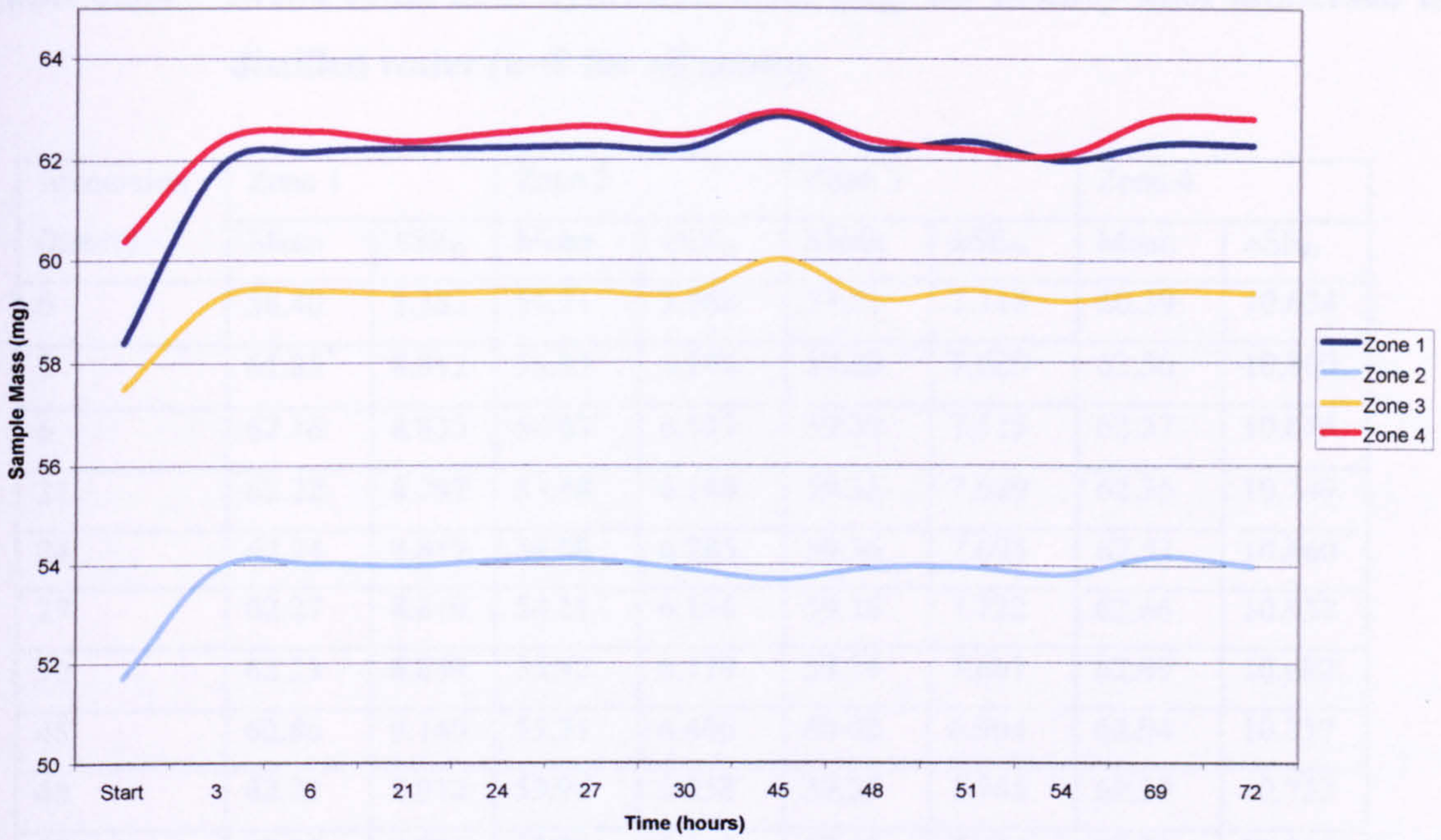


Figure 6.3.11 Fluctuation in mass (mg) of zonal *stratum medium* samples of donkey hoof over a 72-hour immersion period in water (n=8 for all zones and HWD)

There was also a significant interaction between the immersion period and hoof zone, Wilks Lambda = 0.083, F (36, 51) = 1.872, $p < 0.05$, partial eta squared = 0.564, which indicates that the zone also has a large effect on hoof sample mass measurement (Figure 6.3.11 and Table 6.3.4). Zone 1 gained significantly more mass over time than zone 2 ($p < 0.05$), zone 3 ($p < 0.001$), and zone 4 ($p < 0.005$). Absolute sample mass measurements are shown in Table 6.3.4.

It is evident from Figure 6.3.11 that the majority of the gain in mass, relative to original sample mass for all four zonal hoof samples, for the 72-hour immersion period in water occurred within the first 3 hours of immersion.

Table 6.3.4 Mean zonal hoof hydration mass (mg) for donkey hoof immersed in distilled water (n=8 for all zones)

Immersion (hours)	Zone 1		Zone 2		Zone 3		Zone 4	
	Mean	$\pm SE_D$	Mean	$\pm SE_D$	Mean	$\pm SE_D$	Mean	$\pm SE_D$
0	58.40	8.582	51.71	5.968	57.51	7.118	60.39	10.634
3	61.85	8.912	53.93	6.196	59.26	7.620	62.36	10.860
6	62.16	8.835	54.03	6.143	59.39	7.513	62.57	10.675
21	62.22	8.797	53.98	6.198	59.33	7.629	62.36	10.749
24	62.24	8.817	54.08	6.285	59.36	7.695	62.53	10.860
27	62.27	8.810	54.11	6.176	59.36	7.722	62.66	10.937
30	62.23	8.889	53.92	6.179	59.34	7.661	62.49	10.692
45	62.86	9.140	53.71	6.406	60.02	6.904	62.94	10.317
48	62.22	8.912	53.95	6.258	59.23	7.765	62.39	10.752
51	62.37	8.881	53.96	6.245	59.40	7.794	62.21	11.013
54	61.99	8.768	53.82	6.213	59.18	7.675	62.10	11.043
69	62.31	8.824	54.18	6.270	59.39	7.677	62.82	10.854
72	62.29	8.861	54.00	6.257	59.36	7.735	62.81	10.727

6.3.6 Difference between parameter changes in zones

Mixed between-within subjects analyses of variance were also undertaken to assess the impact of hoof wall zone on the measured parameters during the immersion period. The main effect comparing the zones was highly significant, Wilks Lambda = 0.795, F (3, 362) = 31.186, $p < 0.001$, partial eta squared = 0.205, which indicates that the zone has a large effect on the changes in the parameters measured during the immersion period. There was also a significant interaction between the hoof wall zone and the parameter measured, Wilks Lambda = 0.632, F (9, 881) = 20.326, $p < 0.001$, partial eta squared = 0.5142. This was substantiated by *post hoc* analysis using the Scheffe test, as significant differences ($p < 0.001$) were seen between zone 1 and 3 and 4, and also between zone 2 and 3 and 4.

6.4 Discussion

6.4.1 Donkey hoof isotherm

At 100%RH the desorption isotherm (Figure 6.3.6) shows zone 1 considerable lower in moisture content, at 24% than zones 2 (33%), 3 (37%) and 4 (40%). This trend continues down to 86%RH with zones 3 and 4 maintaining similar isotherm curves to each other, while zones 1 and 2 are noticeably lower.

Bertram & Gosline (1987) calculated that an equivalent to *in vivo* moisture content was obtained at 75%RH for horse hoof, although Hopegood found it to be nearer 100%RH for donkey hoof. Studies for this thesis have shown mean donkey hoof *in vivo* moisture contents at midline dead centre to be 22.2%, 29.8%, 37.5% and 39.4% for zones 1, 2, 3 and 4 respectively (from Chapter 3). These would correspond to 96, 97.5, 99.8 and 99.8RH% on the desorption isotherm in Figure 6.3.6, so the findings of this study for reproduction of zonal *in vivo* moisture contents are in agreement with the findings of Hopegood (2002) for full hoof wall depth samples.

Just above 80%RH the zonal isotherms of zones 2, 3 and 4 cross over, and zones 1 and 2 are shallower isotherms than those of zones 3 and 4. There is a distinct division between the lines for the outer two zones 1 and 2; and the inner two zones 3 and 4 between 8%RH and 80%RH (Figure 6.3.6). These differences in isothermal behaviour are perhaps representative of the differences in the hygroscopic properties of the four hoof wall zones. As has previously been discussed in Section 1.2.12, there are significant differences in hoof wall morphology and tubule density across the zones of the hoof wall depth. These may have a direct impact on the nature and quantity of water binding and absorption sites. The ultrastructure of the hoof horn in each zone needs further investigation to confirm this hypothesis.

Given the findings of D'Arcy & Watt (1980), the shallow nature of the isotherms for zones 1 and 2 would indicate that those zones would have less capacity for water binding with peptide chains and subsequent layering of loosely held water, filling voids,

at higher RH%. It may be that the water that is absorbed at higher RH% is contained within the intertubular horn, as there is a lower tubule density within zones 3 and 4 (Hopegood, 2002), and therefore a higher proportion of intertubular material, when compared with zones 1 and 2 (see Section 1.2.12).

Charalambopoulou *et al.*, (1998) investigated water sorption on porcine *stratum corneum* using very small angle neutron scattering, and proposed a swelling mechanism for water sorption which they associated with alterations in the RH% of the environment (Figure 6.4.1).

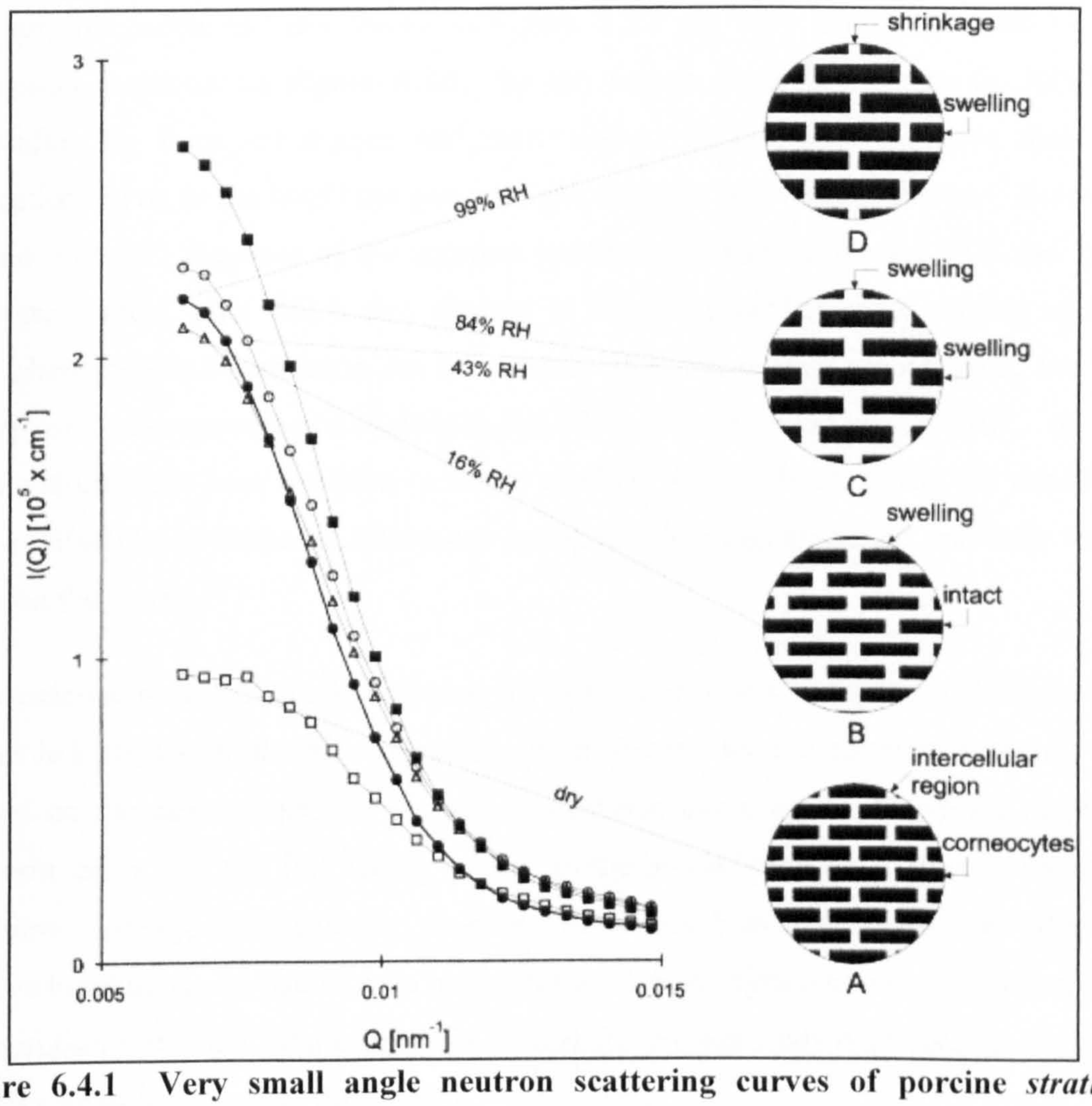


Figure 6.4.1 **Very small angle neutron scattering curves of porcine *stratum corneum* at various RH% and the proposed mechanism of water sorption (Charalambopoulou *et al.*, 1998)**

They attributed the water to swelling of corneocytes and intercellular material, and it could be that a similar mechanism exists in equid hoof horn, whereby the tubular and intertubular horn absorbs water according to changes in RH%. This is an area for further investigation.

Within the first section of the donkey hoof wall sorption and desorption isotherms (Figures 6.3.2 to 6.3.5), , all the zones have similar curves up to 12% RH, which would indicate that they have similar proportions of highly active binding sites available to water.

The sorption isotherm lines shown in Figure 6.3.7 are very similar to those of the desorption isotherm in Figure 6.3.6, for the region from 0%RH up to 50%RH. Thereafter the lines are steeper and more uneven in the sorption curve than the desorption curve, as the hoof horn gains weight at higher relative humidities. Between 53 and 64% RH the lines of the sorption isotherm are similar around 17.5 and 20% moisture content, after which they diverge to follow upward lines of varying angles. This phenomenon is similar to the one displayed in the desorption isotherm, but the desorption lines converge at a slightly higher RH% of between 86 and 76%RH. As the keratin structure in hoof is similar to that in wool (D'Arcy & Watt, 1970), the variances above 75%RH may indicate differences in capacity for weakly bound and bulk water between the zones.

As mentioned previously in this chapter, the difference between sorption and desorption curves is known as hysteresis. Normally, when desorption and sorption isotherms are plotted on the same graph, the desorption isotherm curve occurs above the sorption isotherm curve for the full length of the isotherm (indicating a higher percentage moisture content), with variable positive differences between the two at different relative humidities. As can be seen in Figures 6.3.1-5 the hysteresis between desorption and sorption isotherms for donkey hoof horn in the present study demonstrates variable negative as well as positive differences.

Figure 6.3.1 for the full hoof wall depth exhibits negative hysteresis along the majority of the isotherm – from approximately 40%RH up to 100 %RH. This effect is smaller in the zonal isotherms (Figures 6.3.2-6.3.5). Between the zones, zone 1 exhibits the greatest negative hysteresis above 50%RH

A line exhibiting no negative hysteresis indicates that the moisture content of the sample undergoing sorption is above that of the same sample undergoing desorption. The negative hysteresis seen in Figures 6.3.1-6.3.5 indicates that the samples undergoing sorption had unexpectedly absorbed more water at the relative humidities, than the same sample had previously held at the same relative humidities when it was undergoing desorption. This may be due to some kind of structural damage which could have occurred at the lower relative humidities. One possible cause is that the 0%RH condition was achieved through the drying of samples with phosphorous pentoxide. If this created protein denaturation, it could explain why more water could subsequently enter the structure of the hoof upon rehydration.

In addition, in the current study, samples were fully hydrated by soaking in distilled water for one week prior to starting the desorption isotherm scale. This is in contrast to Hopegood (2002), who states that desorption samples commenced desorption from *in vivo* moisture contents, and were not fully hydrated before starting the experiment.

Hopegood (2002) also seems to have used different samples for sorption and desorption studies, although this is not definitely clear in her thesis. In the current study the same samples were used for both desorption and sorption isotherms. While intended to produce more valid comparisons, samples were left to equilibrate and weighed weekly, so it may be that the extended period of time which the samples were kept at the lower relative humidities had caused irreversible structural changes in the hoof horn structure.

From this study, and that of Hopegood (2002), *in vivo* moisture contents are projected to occur at 96-100% RH. Salts type cabinets are suitable for long-term fixed condition testing, but it is feasible that this lengthy exposure of the hoof material to humidities considerably lower than those experienced at *in vivo* moisture contents could result in damage to the hoof horn structure, as *in vivo* hoof moisture is generally maintained either by absorption of water from the external environment, or diffusion of water

through from the internal tissues of the hoof. The effect of drying on the tubules and intertubular material can be seen in the full hoof wall depth sample in Figure 6.4.2.

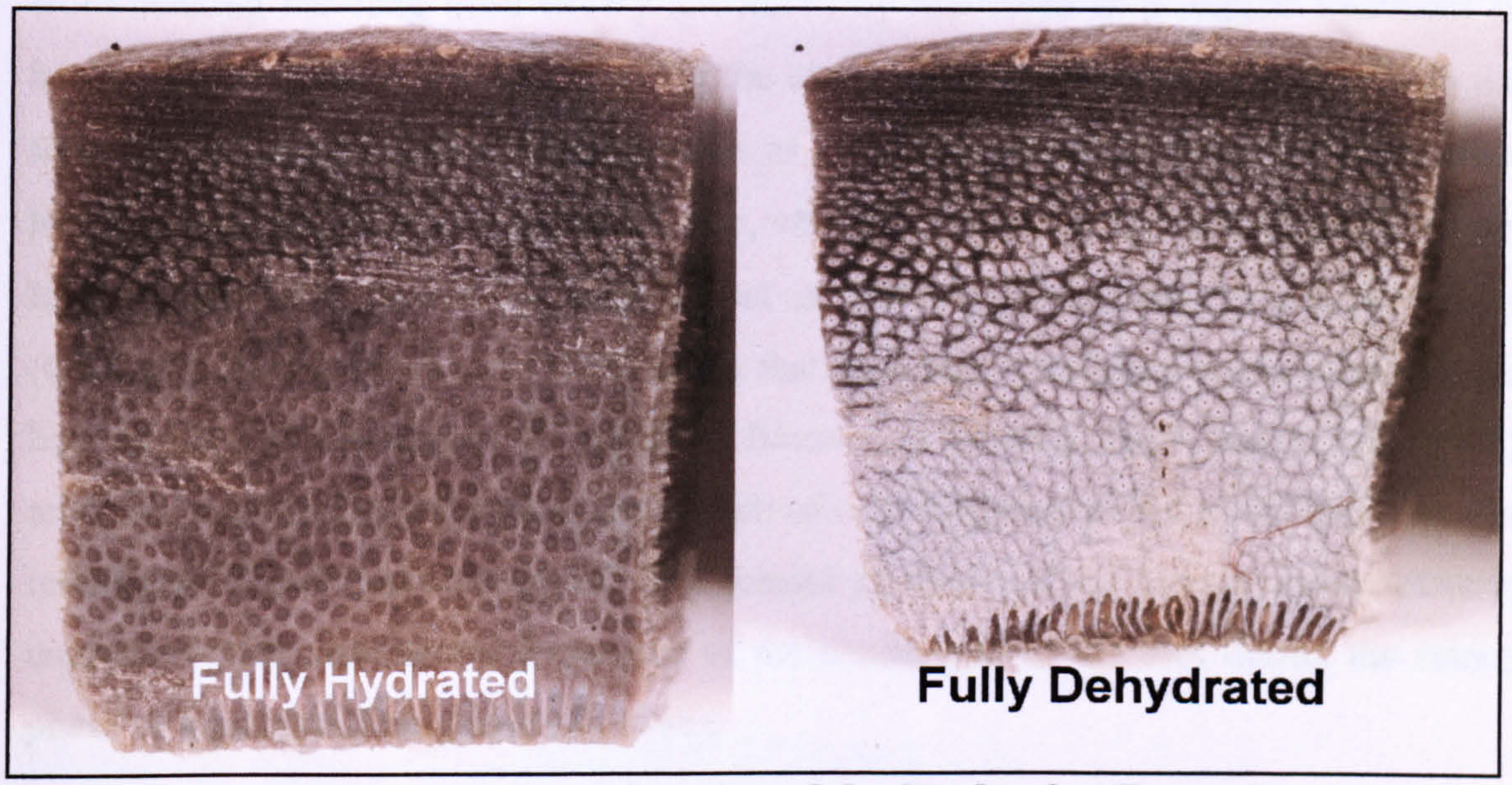


Figure 6.4.2 Fully hydrated and dehydrated donkey hoof wall sample

Care needs to be taken when extrapolating data from *in vitro* experiments and relating them to *in vivo* conditions. *In vivo* only the outside of the *stratum externum* is exposed to water, and water must diffuse through the zones of the *stratum medium* in order to hydrate the hoof. However, because the findings of this study are similar for all hoof zones, including the external zone 1, these results could relate to *in vivo* situations, although further investigation of absorption of the hoof *in vivo* is required to substantiate this assumption.

Kapsalis (1981) indicated that hysteresis, in its positive form, could be considered to be a built in mechanism against extremes, such as loss of water. Hopegood (2002) indicated that this may be extremely useful for donkeys living in hot climates. However, where the donkey hooves are exposed to low relative humidities, or may become dehydrated for extended periods of time through deficiencies in the mechanism which exists for maintaining hoof moisture from the internal vascular structures, negative hysteresis could indicate damage to the hoof structure has occurred.

6.4.2 Immersion in distilled water

Hoof soaking has long been widely advocated by horsemen as a regular treatment of benefit to the horses' hooves. Soaking the equine foot has played a major role in the treatment of common foot ailments such as abscesses, puncture wounds, corns, foot bruising and laminitis despite little, if any, veterinary research on its therapeutic value. It is questionable if soaking horses' feet in water provides any beneficial effects (O'Grady, 2001). Lambert (1966) noted that too-great a devotion to moisturising the hoof could result in adverse effects Both Bertram & Gosline (1987) and Leach (1980) artificially manipulated the moisture content of equine horn and found that stiffness was reduced at higher moisture levels. The greater pliability of the horn at the quarters is consistent with documented expansion of the caudal part of the foot during the stance phase of the stride (Douglas *et al.*, 1996).

It is essential that an optimum moisture content for plasticising the hoof structure to prevent brittle fracture is maintained, without overhydrating the hoof horn. Overhydration would result in the loss of the keratin fibril structure due to imbibing water much greater than the equilibrium moisture content (bound) content at normal ambient external conditions. Establishing precisely at what moisture content this state occurs is an area for future investigation.

All samples in the current study were of similar dimensions at the start of the soaking period, but they were standardised to eliminate any possible differences, and subsequent losses or gains were calculated as mg per 100mg for mass measurements, or mm per 100mm for distance measurements

Multivariate analysis showed that the immersion period in distilled water had a significant effect on the width and mass dimensions of the hoof horn ($p < 0.05$). However, there were no significant differences ($p > 0.05$) between measurements from 3-72 hours. This would signify that the hoof, although the hoof gains mass and width as a result of absorbing water within the first three hours, it does not deform significantly from its original hoof wall depth structure/dimensions. The effect of soaking of hooves

in water on the overall dimensions of the hoof wall is a subject for future *in vivo* investigation.

Despite the variability of the data, when individual zones are studied (Figure 6.3.8-6.3.11), they show some interesting trends. Zone 1, the outer hoof wall, gains over 5mm/100mm width and 6mg/100mg weight while sample height and length hardly change at all. This would be the equivalent to an increase in the hoof wall circumference. A similar result is shown for zone 2, with a 4mg/100mg increase in mass and a 5mm/100mm increase in width. This would result in an increase in the overall circumference of the hoof in the outer two zones, the direction in which this could be extrapolated to the overall hoof is illustrated by the arrows on the wrapped hoof clipping shown in Figure 6.4.3. The sample used in the soaking trial was a midline dead centre sample

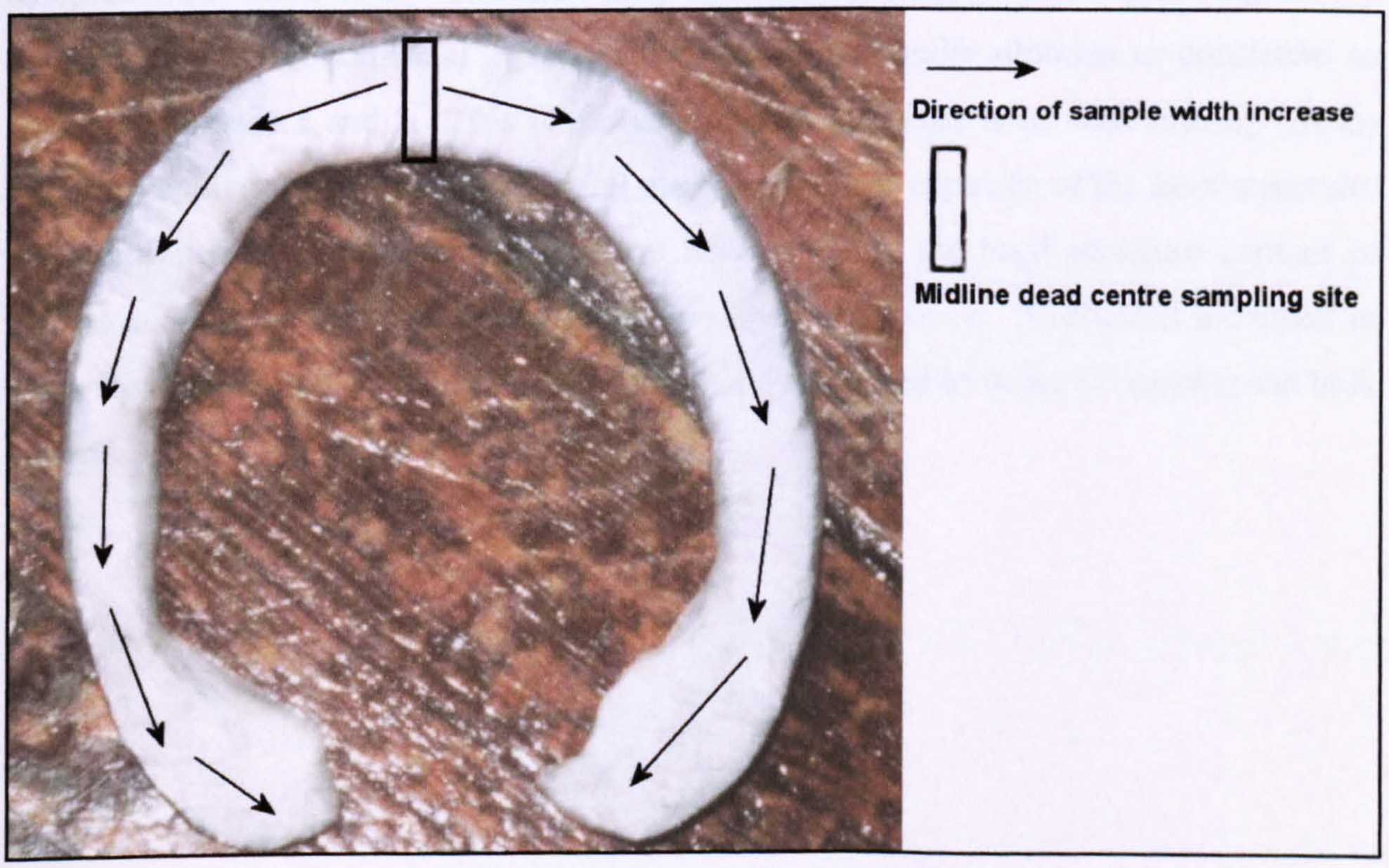


Figure 6.4.3 Direction of increase in sample width illustrated as a hypothetical increase in circumference through the whole length of a wrapped clipping sample

Personal observations of soaked or wet horse hooves *in vivo* have shown that visible swelling of the outer hoof wall does occur, and is especially noticeable around shoe clenches and toe clips. Once hooves have been removed from the water, they lose the excess water in the hoof to the atmosphere, as would be expected from such a hygroscopic material. This presents a problem for farriers if they have to shoe a horse with wet hooves, as once the hooves have dried out and contracted, the clenches appear to have risen away from the hoof wall and need tightening again to prevent shoe loss or spreading. Similar observations were made by Moyer (1989) who noted that horses that are subjected to wet grass in the morning and hot dry conditions later in the day often lose shoes easily.

The inner two zones 3 and 4 do not display such a marked increase in sample width (Figures 6.3.10) (with the exception of an outlier at 21 hours for zone 4). Even though samples from these zones increase in mean mass by 3mg/100mg and 3.5mg/100mg respectively, the dimensional increases are not as profoundly obvious or consistent as they are in zones 1 and 2. This is probably due to the inner hoof wall starting (fresh) moisture content being closer to the full moisture content capacity of the hoof structure. As mentioned in the isotherm discussion Section 6.4.1, the hoof moisture content *in vivo* is at, or near, 99%RH for the inner two hoof wall zones. Additional increases in mass for zones 3 and 4 are likely, therefore, to be attributed to water absorption via bulk water.

6.5 Conclusions

- Donkey hoof wall zones 1 and 2 displayed similar desorption isotherms and similar sorption isotherms, as did donkey hoof wall zones 3 and 4. This would indicate an 'inner' and 'outer' hoof wall depth split with regard to hydration properties of the hoof horn, as there were noticeable differences between the desorption and sorption isotherms for the outer hoof wall zones (1 and 2), and the inner hoof wall zones (3 and 4).

- Negative hysteresis was a characteristic of all the desorption/sorption isotherms and may have been an artefact of the drying process used to obtain 0%RH or the extended period the samples were exposed to lower relative humidities.

- Donkey hoof horn samples (of equivalent measurements to those in this study) can be immersed in distilled water and reach equilibrium mass within 3hrs of first being immersed.

- Full donkey hoof wall depth and zonal hoof samples gained between 3 and 6mg/100mg mass on immersion in distilled water.

- Donkey hoof wall zones 1 & 2 consistently displayed a 5mm/100mm increase in sample width on immersion in distilled water, which would equate to swelling of the outer hoof wall on soaking in water *in vivo*.

- Little or no effect was recorded on the sample height or length, through immersion in distilled water. This would equate to hoof wall height and hoof circumference on soaking *in vivo*.

CHAPTER 7 – ENUMERATED CONCLUSIONS

The work described in this thesis has made original contributions to scientific endeavour and to knowledge in the following ways:

7.1 Hoof Moisture

7.1.1 The full hoof wall depth MC% decreased, from the winter months to the summer months for all three species, and then increased once again towards the autumn. The decrease in hoof MC% was significant ($p < 0.05$) between the winter and summer months in donkeys and ponies, but not in mules. The lowest hoof MC% was seen in May in mules and donkeys, and July in ponies. There is clearly some seasonal effect on hoof MC%, be it due to a physiological, nutritional or environmental factor, or indeed a combination of factors.

7.1.2 As with the full hoof wall depth MC%, the zonal hoof MC% was higher in the winter months than the summer months for all three species. The decrease in zone 1 hoof MC% was significant ($p < 0.05$) between the winter and summer months in donkey hoof, but not in mule or pony hoof. In zone 2 the decrease in hoof MC% was significant ($p < 0.05$) between the winter and summer months in both donkey and pony hoof, but not in mule hoof. Significant decreases ($p < 0.05$) in zone 3 and 4 MC% were seen between the winter and summer months in the hooves of all three species. It appears that the inner hoof wall zone MC% is more sensitive to the effects of season than the outer zones, which may be due to physiological or nutritional

factors, rather than being influenced solely by the external environmental humidity or precipitation.

7.1.3 Donkeys had significantly higher ($p < 0.05$) hoof wall depth MC% than both mules and ponies for all months except July, where the difference was only significant between donkey and mule hoof MC%. Mule hoof wall depth MC% was significantly higher ($p < 0.05$) than pony from February to September. Mule hoof wall depth MC% was on average 2.55% lower than that of the donkey hoof and 5.18% higher than that of pony hoof throughout the seasons. This indicates that the moisture characteristics of mule hoof horn are more like those of donkey, rather than pony hoof, which could have implications for the management of mules in temperate environments

7.1.4 Donkeys had significantly higher ($p < 0.05$) zonal hoof MC% than ponies for all months for all zones. Mule hoof MC% had significantly higher ($p < 0.05$) zonal MC% than pony for all months for zones 2 and 3; and all months except February for zone 4. The differences between donkey and mule hoof zonal MC% were not as widespread and were confined to December and May. Donkey hoof had significantly higher ($p < 0.05$) zone 1 and 2 hoof MC% than mule hoof for December, and significantly higher ($p < 0.05$) zone 2, 3 and 4 hoof MC% than mule hoof for May. This comparison substantiates the argument that mule hoof horn is closer in MC% distribution within the hoof horn to that of the donkey rather than the pony.

7.1.5 There were no significant differences ($p > 0.05$) in full hoof wall depth MC% between locations around the hoof for all three species, although both medial and lateral quarters and heels had higher hoof MC% than the midline

dead centre for donkeys, mules and ponies. So although this increased MC% may be a major contributory factor in higher elasticity levels in the heels, it is not a significant increase ($p > 0.05$).

7.1.6 There were no significant differences ($p > 0.05$) in zonal hoof MC% between locations around the hoof for all three species. As with the full hoof wall depth MC%, there was an insignificant increase in MC% from the midline dead centre to the heels, but this was not uniform throughout the zones. Both the medial and lateral quarters and heels had higher hoof MC% than the midline dead centre for donkeys, mules and ponies in zones 1 to 3, but this was not the case for zone 4 with no particular circumferential MC% trend shown in that zone.

7.1.7 When the mean zonal hoof MC% was compared, it significantly increased ($p < 0.001$) in an anterior-posterior direction towards the internal hoof structures, away from the outer hoof wall for donkeys, mules and ponies. The increase in hoof MC% from external zone 1 to internal zone 4 was 12.06% for donkeys, 12.77% for mules and 11.49% for ponies. The manner in which the interzonal hoof MC% increases occurred across the depth of the hoof wall were even in the pony hoof, but donkey and mule exhibited large increases between zones 1 and 2 hoof MC%, and 2 and 3 hoof MC%, and then a smaller increase between zone 3 and 4 hoof MC%. This is another comparison which further substantiates the argument that mule hoof horn is closer in MC% composition to the hoof horn of the donkey than that of the pony.

7.2 Hoof bound water content

7.2.1 The full hoof wall depth BWC (mg/mg dry matter) of donkeys, mules and ponies tends to be at its lowest in summer, with hoof BWC (mg/mg dry matter) in July and May being lower than in February and September.

7.2.2 The zonal hoof wall bound water content (mg/mg dry matter) of donkeys, mules and ponies varies minimally between months for zone 1 and 2, however, zonal hoof wall BWC (mg/mg dry matter) for all species varies to a larger extent between months in zones 3 and 4.

7.2.3 When zonal hoof wall BWC (mg/mg dry matter) was compared, it increased in an anterior-posterior direction towards the internal hoof structures, away from the outer hoof wall for all species. The rise in hoof BWC (mg/mg dry matter) from external zone 1 to internal zone 4 was on average 0.400 mg/mg for donkeys, 0.357 mg/mg for mules and 0.219 mg/mg for ponies.

7.2.4 Ponies showed a characteristic anterior-posterior hoof bound water content (mg/mg dry matter) gradient with a gradual increase in BWC from zone 1 to zone 3 and then a larger increase between zones 3 and 4. The mule hoof BWC gradient was similar to ponies between zones 1 and 2 and then showed distinct divisions with larger increases between zones 2 and 3, and zones 3 and 4, which was more characteristic of the donkey hoof BWC gradient. This may indicate that the water binding characteristics of the mule hoof are some kind of hybrid between those of the pony and the donkey.

7.2.5 There is little variation in the zone 1 hoof BWC of all species between months. The inner zones 3 and 4 in mule and pony hoof horn appear to have lower hoof BWC in the summer months, whereas the donkey zone 3 and 4 hoof BWC increases in the summer months.

7.3 Hoof Growth and Wear

7.3.1 In pony and mule hoof there was no significant difference ($p > 0.05$) between hoof growth rates (mm/day) for between months. Donkey hoof experienced a significant drop in hoof growth rate between September and November ($p < 0.05$)

7.3.2 The mules and ponies displayed cyclical spurts in hoof growth rates, with increased growth rates in February, May and September. Donkeys, mules and ponies displayed the lowest hoof growth rate (mm/day) in the month of November.

7.3.3 The mule hoof wear rate was higher than for both donkey and pony at all locations, however the multivariate analysis only produced a significant interaction ($p < 0.001$) between species hoof wear rate (mm/day) and the medial heel hoof wall location.

7.3.4 In all three species the midline dead centre hoof wear rate was significantly higher ($p < 0.05$ to $p < 0.001$) than the quarters and heels

7.3.5 General linear model analyses carried out on the wall location hoof wear data produced variable results and did not show any strong trends, although the medial heel hoof wear rate produced many significant interactions ($p < 0.05$) with the month, species, farm and gender factors, when the other hoof wall locations did not.

7.3.6 There was a significant effect ($p < 0.05$) of hoof wall location on net growth/wear (mm/day) for the midline dead centre sampling site for all species, which was evident as a high net hoof wear in February

7.3.7 The months of February, April and November have the lowest net hoof growth/wear (mm/day) figures for all three species. All species appear to display alternating periods of elevated growth rate (mm/day), followed by elevated wear rate (mm/day), producing cyclical fluctuations in the net hoof growth/wear rates (mm/day).

7.3.8 No significant interaction ($p < 0.05$) was found between net hoof growth/wear rate (mm/day) and species, gender or pigmentation and any of the hoof wall locations. The net hoof growth/wear rate (mm/day) interacted significantly with the farm for the midline dead centre ($p < 0.001$) and medial heel ($p < 0.05$) hoof wall locations.

7.3.9 General linear model analysis showed no significant interactions ($p > 0.05$) between hoof replacement time (days) and species, farms, gender, hoof pigment, or hoof wall locations.

7.3.10 Donkey hoof replacement time was greater than that of both mules and ponies due to the slow hoof growth rate (mm/day) of donkey hoof horn.

7.3.11 Donkey, mule and pony all displayed longer hoof replacement times for the medial quarter and heel, compared to the lateral quarter and heel hoof locations, although these differences were not significant ($p > 0.05$).

7.4 Hoof Hydration and Dehydration

7.4.1 Donkey hoof wall zones 1 and 2 displayed similar desorption isotherms and similar sorption isotherms, as did donkey hoof wall zones 3 and 4. This would indicate an 'inner' and 'outer' hoof wall depth split with regard to hydration properties of the hoof horn, as there were noticeable differences between the desorption and sorption isotherms for the outer hoof wall zones (1 and 2), and the inner hoof wall zones (3 and 4).

7.4.2 Negative hysteresis was a characteristic of all the desorption/sorption isotherms and may have been an artefact of the drying process used to obtain

0%RH or the extended period the samples were exposed to lower relative humidities.

7.4.3 Donkey hoof horn samples (of equivalent measurements to those in this study) can be immersed in distilled water and reach equilibrium mass within 3hrs of first being immersed.

7.4.4 Full donkey hoof wall depth and zonal hoof samples gained between 3 and 6mg/100mg mass on immersion in distilled water.

7.4.5 Donkey hoof wall zones 1 & 2 consistently displayed a 5mm/100mm increase in sample width on immersion in distilled water, which would equate to swelling of the outer hoof wall on soaking in water *in vivo*.

7.4.6 Little or no effect was recorded on the sample height or length, through immersion in distilled water. This would equate to hoof wall height and hoof circumference on soaking *in vivo*.

CHAPTER 8 – SUMMARY OF FUTURE RESEARCH DIRECTIONS

8.1 Hoof Moisture Studies

- Further investigation of the microstructure of mule hoof through sectioning of the hoof and histological staining, would be useful in establishing base levels for tubule distribution throughout the hoof, as well as tubular and intertubular horn fractions within the *stratum medium*. This could then be related to the moisture distribution within the mule hoof. This could then be related to the bound and free water distribution within the mule hoof.

- Similarly, a further study could be carried out to ascertain the precise moisture content of the individual tubular and intertubular horn fractions. This would give further knowledge of exactly where within the microstructure of the hoof, the moisture is held. Care would need to be taken to ensure that fresh hoof samples could be analysed quickly, with minimal preparation time, or ideally tested within a controlled environment. This is due to the speed at which moisture is lost from fresh hoof samples when they are exposed to the external atmosphere.

- Analysis of the moisture content of the hooves of lame animals and then comparison of that to the hoof moisture content when the animal is sound may give some indication of the likelihood of lameness based on hoof moisture (%) results. As it has been hypothesised that lameness and disease are more common when hooves have a higher percentage moisture content (Borderas *et al.*, 2004).

- Measurement of weather conditions, including maximum and minimum temperature, rainfall and relative humidity (%) both when samples are taken, and also between sampling occasions could provide information regarding the association between these parameters and hoof moisture (%).

8.2 Hoof Bound and Free Water Studies

- Further studies could be carried out to ascertain the bound and free water content of the individual tubular and intertubular horn fractions. This would further knowledge of exactly where and how, within the microstructure of the hoof, the water is held. Tubular and intertubular horn would need to be prepared ahead of being fully hydrated, to prevent any moisture loss while putting the sample in the pan and crimping the lid prior to analysis.

- Analysis of the bound water content of the hooves of lame animals and then comparison of that to the hoof bound water content when the animal is sound may give some indication of the likelihood of lameness based on hoof bound water content results.

8.3 Hoof Growth and Wear Studies

- Analysis of the calorific intake of the animals could be made to establish if their plane of nutrition has any effect on hoof growth rates of donkey, pony and mule hoof horn.

- Further study could be undertaken to establish whether feed supplementation or other measures could be introduced to increase the rate of horn production to aid the recovery of donkeys admitted to the Sanctuary, as biotin supplementation has been demonstrated to increase hoof growth without increasing the calorific intake in horses.

- Pedometers could be fitted to study animals to establish distances they travelled. This could then be compared to the hoof wear rate of each species. Different terrains could also be investigated, as concrete is known to have an abrasive effect on cattle hoof horn.

- As van der Tol *et al.,* (2005) have stated that horn hardness and elasticity correlate roughly with strength, wear resistance and brittleness in cattle claws; hardness testing of hoof material from donkeys, mules and ponies could be undertaken to establish if there is a correlation between the hardness of the hoof and the rate of wear.

- Further investigation relating to the microstructure of the hoof through sectioning of the hoof and histological staining, to establish tubule

distribution throughout the hoof could be used to explore the hypothesis of Schummer *et al.*, (1981) that tubule density is related to the resistance to wear of the hoof.

- A large percentage of donkeys at the Sanctuary have laminitis. Hoof growth rate of healthy and diseased animals could be compared to establish hoof growth patterns in laminitic donkeys and ponies in a temperate climate. If there was an identifiable growth pattern then it could maybe used as an indicator of laminitis.

8.4 Hoof Hydration and Dehydration Studies

- The relative humidity chamber could be used to condition material prior to bound water analysis using the DSC, to establish at what moisture content the hoof horn demonstrated free water content.

- Advanced scientific instruments which can regulate the environmental conditions to provide high speed humidity changes, such as the Dynamic Vapour Sorption Analyser (Surface Measurement Systems Ltd. London), may be suitable to undertake a series of stepwise experiments to determine exactly which relative humidity any possible hoof keratin structural damage due to excessive desiccation may start to occur.

- A further study could be carried out to ascertain just how long the hoof material has to be exposed to the desiccating effects of the low relative humidity, before any damage to the keratin structure of the hoof horn may start to occur.

- Sorption and desorption experiments should also be carried out for pony and mule hoof horn to see whether there are, indeed, species differences in the isotherms produced by the sorption of water into or out of the hoof horn.

- Possible reasons for the desorption isothermal similarity between Zones 1 and 2, and also between Zones 3 and 4 could be investigated further by carrying out histological studies on the mircostructure of the hoof. This could establish not only the tubule distribution throughout the hoof, but also the relative fractions of tubular and intertubular horn within each of the four zones. This could then be related to the isotherms obtained, and may subsequently offer possible reasons for the differences between the desorption isotherms for the outer hoof wall zones and the inner hoof wall zones respectively.

- On immersion in distilled water, more frequent measurements could be taken to determine the uptake of water by donkey hoof horn in the first 3 hours of immersion. As there was no significant difference ($p < 0.05$) between any of the measurements from 3 hours post immersion to 72 hours post immersion, and the majority of the water uptake occurred in the first three hours, more frequent measurements could ascertain the rate of water uptake per hour. It may be possible to take measurements and weigh samples every 15minutes, but care must be taken not to interrupt the natural water uptake of the sample.

- Whole hoof wall water uptake could be measured *in vivo* by immersing a whole hoof cadaver in distilled water. This would provide information on the effect water uptake has on the whole intact hoof structure, as would happen if the horse were to live in marshland conditions. If the other surfaces of the foot, such as the sole, frog and heel, were to be sealed to prevent water uptake or loss from those surfaces then the water uptake from the hoof wall alone could be ascertained.

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APPENDIX 1 - Whole trial data set mean values hoof moisture content

Mean values all species lateral heel hoof moisture content (%)

Zone 1	Donkey	n	±SE_D	Mule	n	±SE_D	Pony	n	±SE_D
December	32.21	6	1.778	24.50	7	3.217	22.96	8	1.408
February	28.60	4	2.755	26.79	7	3.426	24.04	4	1.184
May	25.69	5	4.834	23.96	6	2.138	21.29	7	1.711
July	27.67	7	3.718	27.70	3	2.768	21.48	8	3.383
September	27.58	7	3.724	24.87	8	2.081	23.14	10	1.748

Zone 2	Donkey	n	±SE_D	Mule	n	±SE_D	Pony	n	±SE_D
December	38.76	5	2.038	34.40	8	2.232	30.61	8	1.431
February	34.78	9	3.850	35.06	7	2.575	30.34	4	1.725
May	33.30	5	4.412	32.26	6	2.666	27.18	7	4.636
July	33.19	8	2.328	33.07	4	2.999	25.59	8	4.019
September	34.33	7	3.486	32.35	9	1.805	28.42	10	2.273

Zone 3	Donkey	n	±SE_D	Mule	n	±SE_D	Pony	n	±SE_D
December	41.20	4	1.830	37.95	8	1.488	34.11	8	2.632
February	39.12	9	1.405	38.55	7	1.973	35.07	4	1.573
May	36.64	5	1.750	33.91	6	3.027	28.47	7	4.749
July	37.03	8	1.582	36.30	4	2.885	29.74	8	5.891
September	37.06	7	2.063	34.80	9	2.732	32.58	10	2.681

Zone 4	Donkey	N	±SE_D	Mule	n	±SE_D	Pony	n	±SE_D
December	42.02	2	1.353	38.19	5	1.409	38.24	1	n/a
February	40.28	7	1.661	38.69	3	1.744	36.05	2	1.485
May	37.02	5	1.514	32.52	6	2.506	30.62	1	n/a
July	36.97	8	1.399	37.76	4	2.822	30.86	3	5.727
September	37.18	6	2.387	35.66	8	1.742	32.65	6	3.451

Mean values all species lateral quarter hoof moisture content (%)

Zone 1	Donkey	n	±SE$_D$	Mule	n	±SE$_D$	Pony	n	±SE$_D$
December	28.65	2	2.890	23.94	4	2.804	20.87	4	1.441
February	24.36	4	1.776	22.92	5	1.920	21.48	4	1.401
May	22.83	5	1.721	22.45	5	4.816	19.07	5	2.009
July	24.97	6	3.763	22.52	4	1.147	19.38	5	2.176
September	25.07	9	4.532	24.78	7	5.477	20.64	7	1.593
Zone 2	Donkey	n	±SE$_D$	Mule	n	±SE$_D$	Pony	n	±SE$_D$
December	38.38	2	0.932	31.69	5	4.621	26.33	4	2.025
February	32.88	4	2.591	29.75	5	2.361	24.99	4	1.358
May	32.06	5	2.948	27.53	5	3.962	21.72	5	2.942
July	30.46	6	1.959	28.54	4	3.239	22.24	5	2.162
September	32.37	9	3.672	29.38	8	2.074	23.88	8	1.877
Zone 3	Donkey	n	±SE$_D$	Mule	n	±SE$_D$	Pony	n	±SE$_D$
December	41.11	2	1.968	36.65	5	1.990	29.67	4	5.946
February	39.01	4	0.827	36.75	5	1.526	31.37	4	1.933
May	37.44	5	0.839	34.02	5	3.327	26.31	5	4.377
July	36.60	6	1.375	34.03	4	2.788	25.39	5	1.796
September	37.34	9	2.045	34.81	8	2.732	28.34	8	4.232
Zone 4	Donkey	n	±SE$_D$	Mule	n	±SE$_D$	Pony	n	±SE$_D$
December	n/a	0	n/a	39.40	4	2.926	34.94	3	5.087
February	40.64	4	1.092	38.56	5	1.118	37.28	4	2.585
May	37.93	5	0.446	34.38	5	2.269	29.97	3	0.261
July	37.49	6	2.046	35.67	4	1.071	30.76	5	3.602
September	38.13	9	2.355	35.60	8	2.639	33.79	8	3.400

Mean values for all species midline dead centre hoof moisture content (%)

Zone 1	Donkey	n	±SE_D	Mule	n	±SE_D	Pony	n	±SE_D
December	24.19	2	0.345	21.11	5	1.643	21.02	4	1.723
February	23.22	4	2.266	23.22	5	4.864	20.29	6	2.514
May	23.01	7	3.288	19.87	7	1.557	18.67	7	1.807
July	23.15	5	6.528	21.08	5	2.050	19.79	6	4.191
September	21.75	10	1.859	20.35	8	1.824	21.34	9	2.824
Zone 2	Donkey	n	±SE_D	Mule	n	±SE_D	Pony	n	±SE_D
December	29.42	2	0.536	26.75	6	2.437	23.67	4	0.944
February	30.24	4	6.475	25.70	5	1.043	24.74	6	2.187
May	30.78	7	3.273	23.86	7	2.179	20.85	7	2.040
July	27.22	5	1.212	25.02	5	1.865	21.50	6	3.709
September	28.91	10	1.755	25.23	9	2.570	23.17	9	1.067
Zone 3	Donkey	n	±SE_D	Mule	n	±SE_D	Pony	n	±SE_D
December	37.90	2	0.758	35.97	6	1.138	31.49	4	5.135
February	39.43	4	1.048	35.19	5	0.704	30.32	6	2.520
May	36.53	7	1.180	32.09	7	2.916	23.77	7	2.286
July	36.21	5	0.992	33.27	5	2.908	24.43	6	4.725
September	36.56	10	1.706	34.46	9	2.571	26.95	9	2.678
Zone 4	Donkey	n	±SE_D	Mule	n	±SE_D	Pony	n	±SE_D
December	41.03	2	0.517	37.82	5	1.005	36.36	3	2.778
February	41.09	3	0.384	38.37	5	1.697	36.43	6	1.753
May	37.42	7	1.518	34.91	7	2.681	29.24	7	2.731
July	38.13	5	1.117	38.07	5	1.558	30.60	6	5.382
September	38.94	10	1.580	37.48	10	3.234	33.60	9	3.167

Mean values all species medial quarter hoof moisture content (%)

Zone 1	Donkey	n	±SE_D	Mule	n	±SE_D	Pony	n	±SE_D
December	24.99	6	1.481	21.45	4	0.183	20.95	4	0.867
February	23.67	5	2.475	22.29	7	1.948	22.44	5	2.491
May	23.10	7	2.754	21.80	7	0.907	18.94	5	1.776
July	24.91	5	2.415	24.22	7	2.597	19.11	4	2.824
September	24.67	8	1.936	22.03	7	1.273	20.52	7	1.507
Zone 2	Donkey	n	±SE_D	Mule	n	±SE_D	Pony	n	±SE_D
December	31.40	5	1.873	29.58	5	1.292	26.58	5	3.231
February	31.81	6	2.229	29.07	7	1.716	25.28	5	2.345
May	30.48	7	3.836	27.96	7	1.288	22.39	5	2.589
July	28.87	6	2.290	28.74	7	3.453	21.76	4	3.481
September	31.33	8	1.807	28.52	8	3.385	23.55	7	2.103
Zone 3	Donkey	n	±SE_D	Mule	n	±SE_D	Pony	n	±SE_D
December	36.92	5	1.634	37.21	5	0.950	33.38	5	3.016
February	37.38	6	1.687	36.06	7	1.476	30.24	5	3.366
May	35.87	7	2.332	30.26	7	1.498	26.34	5	3.872
July	35.15	6	1.586	34.43	7	1.992	25.22	4	3.657
September	36.43	8	1.922	34.69	8	2.622	28.12	7	3.360
Zone 4	Donkey	n	±SE_D	Mule	n	±SE_D	Pony	n	±SE_D
December	39.03	3	1.613	38.30	4	0.785	39.94	3	2.815
February	39.94	6	1.036	38.51	7	1.625	35.79	4	1.574
May	37.07	7	1.004	34.77	7	1.801	28.72	4	5.017
July	38.01	6	2.660	36.55	7	1.496	30.82	4	4.603
September	38.11	8	1.352	36.96	9	1.706	32.85	6	3.329

Mean values for all species medial heel hoof moisture content (%)

Zone 1	Donkey	n	±SE_D	Mule	n	±SE_D	Pony	n	±SE_D
December	30.63	9	4.656	24.48	5	2.706	22.00	6	1.240
February	26.28	6	1.291	26.22	7	3.167	23.88	6	1.461
May	28.39	7	3.857	24.51	8	1.617	22.25	6	1.181
July	26.02	2	8.987	24.98	5	0.656	21.64	6	3.021
September	26.26	4	4.877	25.84	9	2.165	22.28	9	0.921
Zone 2	Donkey	n	±SE_D	Mule	n	±SE_D	Pony	n	±SE_D
December	37.45	9	3.184	33.44	5	2.359	29.66	6	1.552
February	33.68	10	1.942	33.68	9	3.021	29.22	6	2.697
May	34.43	8	3.493	27.70	8	3.768	27.19	6	2.712
July	30.93	6	4.209	30.33	6	1.919	27.60	6	3.101
September	32.44	10	2.700	33.08	9	2.232	27.54	9	1.640
Zone 3	Donkey	n	±SE_D	Mule	n	±SE_D	Pony	n	±SE_D
December	38.62	8	2.432	36.95	4	2.086	35.69	4	3.173
February	39.69	10	1.065	37.90	9	1.009	35.16	6	2.163
May	37.10	8	1.404	34.49	8	1.738	29.21	6	2.072
July	36.70	6	1.808	34.55	6	2.673	29.37	6	3.014
September	36.29	10	2.752	36.04	8	2.466	31.85	9	2.794
Zone 4	Donkey	n	±SE_D	Mule	n	±SE_D	Pony	n	±SE_D
December	37.82	4	5.697	37.12	1	n/a	35.93	1	n/a
February	41.46	7	2.360	38.81	3	0.730	38.53	3	3.367
May	36.43	8	1.839	34.35	7	2.327	32.61	1	n/a
July	37.29	6	1.226	36.44	5	3.508	31.59	3	3.552
September	37.55	9	2.163	36.75	9	1.762	34.15	5	4.424

APPENDIX 2 - Mean hoof bound water content (mg/mg dry matter) all species

Zone 1	Donkey	n	±SE$_D$	Mule	n	±SE$_D$	Pony	n	±SE$_D$
December	0.437	3	0.040	0.314	5	0.043	0.315	2	0.049
February	-	0	-	0.332	5	0.016	0.308	6	0.026
May	0.357	3	0.011	0.356	5	0.044	0.436	7	0.070
July	0.327	4	0.029	0.365	4	0.021	0.347	7	0.047
September	0.428	6	0.090	0.369	7	0.018	0.329	8	0.021

Zone 2	Donkey	n	±SE$_D$	Mule	n	±SE$_D$	Pony	n	±SE$_D$
December	0.650	3	0.017	0.462	5	0.116	0.365	2	0.049
February	-	0	-	0.398	5	0.025	0.388	6	0.039
May	0.460	3	0.044	0.370	5	0.032	0.550	7	0.134
July	0.445	4	0.057	0.385	4	0.038	0.366	7	0.049
September	0.488	6	0.038	0.390	7	0.037	0.359	8	0.024

Zone 3	Donkey	n	±SE$_D$	Mule	n	±SE$_D$	Pony	n	±SE$_D$
December	0.537	3	0.115	0.604	5	0.133	0.565	2	0.092
February	-	0	-	0.604	5	0.061	0.468	6	0.068
May	0.680	3	0.020	0.516	5	0.068	0.466	7	0.043
July	0.635	4	0.052	0.530	4	0.062	0.421	7	0.042
September	0.697	6	0.059	0.579	7	0.080	0.425	8	0.020

Zone 4	Donkey	n	±SE$_D$	Mule	n	±SE$_D$	Pony	n	±SE$_D$
December	0.763	3	0.029	0.716	5	0.109	0.750	2	0.113
February	-	0	-	0.702	5	0.105	0.640	6	0.060
May	0.807	3	0.015	0.696	5	0.032	0.400	7	0.111
July	0.717	4	0.038	0.720	4	0.051	0.557	7	0.072
September	0.838	6	0.145	0.706	7	0.165	0.622	8	0.044

HWD	Donkey	n	±SE$_D$	Mule	n	±SE$_D$	Pony	n	±SE$_D$
December	37.07	3	3.886	37.12	5	n/a	35.93	2	n/a
February	41.46	0	0.892	38.81	5	0.422	38.53	6	1.944
May	36.08	3	0.633	34.35	5	0.879	32.61	7	n/a
July	35.76	4	1.436	36.44	4	1.569	31.59	7	2.051
September	37.55	6	0.721	36.75	7	0.587	34.15	8	1.978

APPENDIX 3 - Whole trial mean values all species hoof growth rate (mm/day)

Lateral Heel	Donkey	n	±SE$_D$	Mule	n	±SE$_D$	Pony	n	±SE$_D$
February	0.169	12	0.036	0.149	12	0.062	0.178	10	0.037
April	0.135	12	0.067	0.189	12	0.076	0.171	10	0.054
May	0.146	12	0.088	0.168	12	0.110	0.141	10	0.096
July	0.149	12	0.035	0.166	12	0.031	0.172	10	0.045
September	0.141	12	0.033	0.178	12	0.039	0.203	10	0.031
November	0.109	12	0.045	0.194	12	0.090	0.180	10	0.044

Lateral Quarter	Donkey	n	±SE$_D$	Mule	n	±SE$_D$	Pony	n	±SE$_D$
February	0.165	12	0.064	0.191	12	0.049	0.191	10	0.036
April	0.126	12	0.050	0.156	12	0.066	0.180	10	0.075
May	0.158	12	0.035	0.216	12	0.064	0.195	10	0.094
July	0.151	12	0.038	0.181	12	0.042	0.158	10	0.051
September	0.133	12	0.053	0.199	12	0.036	0.191	10	0.036
November	0.112	12	0.030	0.160	12	0.053	0.176	10	0.072

Midline Dead Centre	Donkey	n	±SE$_D$	Mule	n	±SE$_D$	Pony	n	±SE$_D$
February	0.165	12	0.064	0.191	12	0.059	0.200	10	0.068
April	0.118	12	0.414	0.163	12	0.063	0.114	10	0.046
May	0.115	12	0.078	0.192	12	0.100	0.166	10	0.097
July	0.142	12	0.044	0.182	12	0.061	0.135	10	0.054
September	0.135	12	0.039	0.221	12	0.039	0.197	10	0.072
November	0.119	12	0.051	0.157	12	0.047	0.159	10	0.049

Medial Quarter	Donkey	n	±SE$_D$	Mule	n	±SE$_D$	Pony	n	±SE$_D$
February	0.185	12	0.076	0.147	12	0.076	0.222	10	0.057
April	0.124	12	0.055	0.161	12	0.102	0.154	10	0.084
May	0.108	12	0.062	0.233	12	0.110	0.154	10	0.078
July	0.133	12	0.040	0.178	12	0.053	0.167	10	0.045
September	0.128	12	0.052	0.217	12	0.062	0.191	10	0.097
November	0.112	12	0.045	0.138	12	0.049	0.132	10	0.051

Medial Heel	Donkey	n	±SE$_D$	Mule	n	±SE$_D$	Pony	n	±SE$_D$
February	0.150	12	0.055	0.163	12	0.046	0.174	10	0.048
April	0.115	12	0.050	0.131	12	0.072	0.147	10	0.107
May	0.220	12	0.098	0.173	12	0.097	0.126	10	0.082
July	0.133	12	0.018	0.164	12	0.056	0.155	10	0.028
September	0.142	12	0.047	0.213	12	0.066	0.191	10	0.089
November	0.090	12	0.043	0.139	12	0.026	0.141	10	0.042

APPENDIX 4 - Whole trial mean values all species hoof wear rate (mm/day)

Lateral Heel	Donkey	n	±SE$_D$	Mule	n	±SE$_D$	Pony	n	±SE$_D$
February	0.050	12	0.041	0.065	11	0.055	0.094	10	0.029
April	0.116	12	0.075	0.135	11	0.080	0.137	10	0.035
May	0.087	12	0.072	0.105	11	0.085	0.109	10	0.049
July	0.058	12	0.068	0.101	11	0.069	0.124	10	0.037
September	0.108	12	0.063	0.106	11	0.053	0.095	10	0.037
November	0.085	12	0.062	0.142	11	0.055	0.155	10	0.063

Lateral Quarter	Donkey	n	±SE$_D$	Mule	n	±SE$_D$	Pony	n	±SE$_D$
February	0.113	12	0.074	0.135	11	0.064	0.090	10	0.054
April	0.106	12	0.056	0.156	11	0.074	0.185	10	0.052
May	0.127	12	0.074	0.180	11	0.089	0.173	10	0.089
July	0.127	12	0.066	0.172	11	0.034	0.119	10	0.063
September	0.137	12	0.035	0.146	11	0.043	0.107	10	0.059
November	0.109	12	0.051	0.144	11	0.067	0.156	10	0.074

Midline Dead Centre	Donkey	n	±SE$_D$	Mule	n	±SE$_D$	Pony	n	±SE$_D$
February	0.319	12	0.218	0.353	11	0.207	0.361	10	0.200
April	0.110	12	0.034	0.152	11	0.054	0.156	10	0.074
May	0.116	12	0.084	0.173	11	0.102	0.113	10	0.108
July	0.105	12	0.065	0.148	11	0.051	0.093	10	0.068
September	0.118	12	0.029	0.167	11	0.029	0.110	10	0.060
November	0.132	12	0.046	0.169	11	0.038	0.163	10	0.073

Medial Quarter	Donkey	n	±SE$_D$	Mule	n	±SE$_D$	Pony	n	±SE$_D$
February	0.057	12	0.052	0.094	11	0.044	0.120	10	0.082
April	0.070	12	0.083	0.106	11	0.063	0.089	10	0.069
May	0.103	12	0.067	0.154	11	0.112	0.061	10	0.063
July	0.083	12	0.067	0.132	11	0.035	0.106	10	0.067
September	0.097	12	0.074	0.143	11	0.056	0.099	10	0.056
November	0.099	12	0.061	0.192	11	0.056	0.147	10	0.065

Medial Heel	Donkey	n	±SE$_D$	Mule	n	±SE$_D$	Pony	n	±SE$_D$
February	0.064	12	0.071	0.058	11	0.039	0.162	10	0.094
April	0.128	12	0.094	0.103	11	0.062	0.081	10	0.064
May	0.126	12	0.117	0.102	11	0.103	0.071	10	0.066
July	0.056	12	0.051	0.087	11	0.065	0.105	10	0.054
September	0.102	12	0.073	0.137	11	0.031	0.084	10	0.034
November	0.067	12	0.061	0.148	11	0.086	0.103	10	0.059

APPENDIX 5 - Whole trial mean values all species net hoof growth/wear rate (mm/day)

Lateral Heel	Donkey	n	±SE_D	Mule	n	±SE_D	Pony	n	±SE_D
February	0.118	12	0.051	0.080	11	0.056	0.085	10	0.040
April	0.019	12	0.081	0.060	11	0.113	0.034	10	0.053
May	0.058	12	0.077	0.070	11	0.089	0.032	10	0.126
July	0.093	12	0.054	0.064	11	0.056	0.047	10	0.053
September	0.032	12	0.070	0.065	11	0.062	0.109	10	0.053
November	0.024	12	0.063	0.054	11	0.096	0.026	10	0.047

Lateral Quarter	Donkey	n	±SE_D	Mule	n	±SE_D	Pony	n	±SE_D
February	0.052	12	0.086	0.049	11	0.049	0.101	10	0.054
April	0.020	12	0.058	0.010	11	0.071	-0.007	10	0.069
May	0.031	12	0.141	0.041	11	0.080	0.021	10	0.123
July	0.023	12	0.063	0.014	11	0.038	0.039	10	0.054
September	-0.005	12	0.057	0.052	11	0.036	0.082	10	0.047
November	0.003	12	0.041	0.016	11	0.042	0.021	10	0.040

Midline Dead Centre	Donkey	n	±SE_D	Mule	n	±SE_D	Pony	n	±SE_D
February	-0.154	12	0.248	-0.146	11	0.226	-0.161	10	0.236
April	0.007	12	0.050	0.016	11	0.066	-0.042	10	0.065
May	-0.008	12	0.075	0.014	11	0.058	0.054	10	0.137
July	0.036	12	0.063	0.032	11	0.034	0.040	10	0.078
September	0.018	12	0.049	0.053	11	0.036	0.088	10	0.066
November	-0.013	12	0.043	-0.127	11	0.039	-0.004	10	0.069

Medial Quarter	Donkey	n	±SE_D	Mule	n	±SE_D	Pony	n	±SE_D
February	0.129	12	0.088	0.061	11	0.089	0.103	10	0.085
April	0.055	12	0.067	0.060	11	0.082	0.064	10	0.052
May	0.006	12	0.095	0.066	11	0.097	0.094	10	0.089
July	0.049	12	0.062	0.042	11	0.052	0.061	10	0.050
September	0.032	12	0.067	0.077	11	0.050	0.091	10	0.056
November	0.013	12	0.070	-0.053	11	0.040	-0.014	10	0.052

Medial Heel	Donkey	n	±SE_D	Mule	n	±SE_D	Pony	n	±SE_D
February	0.085	12	0.083	0.110	11	0.038	0.024	10	0.110
April	-0.142	12	0.099	0.037	11	0.073	0.060	10	0.055
May	0.094	12	0.092	0.063	11	0.116	0.063	10	0.116
July	0.056	12	0.051	0.084	11	0.059	0.060	10	0.054
September	0.041	12	0.077	0.087	11	0.095	0.116	10	0.091
November	0.024	12	0.047	0.003	11	0.080	0.036	10	0.060

APPENDIX 6 - Publications and Conference Proceedings

COLLINS, S.N., WEALLEANS, H., HOPEGOOD, L., LATHAM, R.J., NEWLYN, H.A. AND REILLY, J.D. (2002) Current studies on the donkey hoof. CPD, Medicine and Surgery of the Donkey, BEVA, University of Glasgow

REILLY, J.D. (2007) My work was presented at the BEVA/ Worshipful Company of Farriers Trimming For Soundness Conference. Stoneleigh.